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INNOVATIONS POUR MAÎTRISER LES DANGERS MICROBIOLOGIQUES DANS LA PRODUCTION ET LA TRANSFORMATION DES ALIMENTS EN REGION TROPICALE

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I - Introduction :

Les aliments et l'eau utilisée pour leur production, leur transformation et leur préparation sont des vecteurs potentiels de nombreux dangers microbiologiques et chimiques. Les intoxications ou maladies d'origine alimentaire posent des problèmes économiques et de santé publique importants tant dans les pays développés que dans les pays en développement. De graves et coûteuses maladies et épidémies d'origine alimentaire se sont produites dans les pays développés et continueront de le faire, bien que les systèmes alimentaires et agricoles soient généralement reconnus comme étant sûrs. Les intoxications alimentaires et les maladies diarrhéiques transmises par l'eau tueraient plus de 2 millions de personnes par an, pour la plupart des enfants, dans les pays en développement - un chiffre comparable au nombre de décès annuels causés par le paludisme. A Madagascar, les maladies diarrhéiques représentent 18% du taux de mortalité. Il s'agit de la troisième cause de mortalité après les infections respiratoires (27%) et le paludisme (22%) (WHO, 2006). En 2008, ces maladies ont été la première cause de mortalité chez les enfants de moins de 5 ans à Madagascar (WHO, 2008). Les pouvoirs publics de nombreux pays en développement n'ont pas les moyens d'établir des normes de sécurité sanitaire des aliments et/ou de les faire respecter, en particulier parce que la réduction des risques transmis par les aliments exige des ressources financières et institutionnelles qu'ils n'ont pas.

En climat tropical, les risques de contamination ou d'altération des produits le long de la chaîne alimentaire sont favorisés par la température et l'hygrométrie élevées. Des pertes économiques importantes dans les différentes filières sont directement liées à la dégradation microbiologique des produits frais et au manque de maîtrise des conditions de stockage (durée, humidité, température). Par ailleurs, l'emploi abusif des antibiotiques dans les élevages animaux a été reconnu comme une cause majeure de l'émergence de bactéries résistantes. Par exemple, la résistance de 3 pathogènes majeurs pour l'homme – *Salmonella* spp., *Campylobacter* spp. and *Escherichia coli* – est liée à l'utilisation des antibiotiques chez les animaux. Nous avons choisi de travailler sur ce sujet, dans le domaine de l'aquaculture. En effet, il s'agit d'une des activités agricoles qui connait le plus fort taux de croissance depuis cinquante ans, avec un taux annuel moyen de 8,8%. L'aquaculture dont 50% des produits est échangé à l'échelle internationale, fournit la moitié du poisson consommé dans le monde. Elle est fortement représentée en zone tropicale où la présence des antibiotiques constitue le critère sanitaire le plus fréquemment incriminé dans les alertes sanitaires communautaires (Union

Européenne). En effet cette industrie s'est intensifiée grâce à l'utilisation des antibiotiques car les maladies bactériennes sont un fléau pour ce type d'élevage.
En termes de protection de la santé publique, ICMSF (International Commission on Microbiological Specifications for Foods) a proposé, au niveau international, un schéma de gestion des dangers sanitaires des aliments incluant le concept d'objectifs de sécurité (FSO : Food Safety Objective) identifiant la fréquence ou le niveau d'un danger microbiologique ou chimique, dans un produit alimentaire. Pour garantir l'atteinte de cet objectif de sécurité, il est nécessaire d'établir des objectifs de performance (POs : Performance Objectives) qui correspondent aux niveaux qui doivent être atteints dans les étapes en amont de la consommation des denrées alimentaires. Les mesures de contrôle le long de la chaîne alimentaire doivent assurer que ces niveaux de performance ne sont pas dépassés. Ainsi, FSO et POs doivent être garantis grâce aux bonnes pratiques (BPA, agricoles ; BPH, hygiéniques ; BPF, de fabrication) et à l'HACCP sur lesquels doit être basée la gestion préventive des risques sanitaires, lors des étapes de production et de distribution des aliments. La définition d'un objectif de sécurité se fonde avant tout sur l'appréciation ou l'évaluation du risque de santé publique associé à un aliment, à un procédé, ou à un contaminant particulier.

Dans le contexte global d'intensification des agricultures mondiales, il est attendu de la recherche sur la qualité et sécurité des aliments d'apporter des solutions pour contribuer à (i) **élaborer des systèmes agricoles durables**, (ii) **protéger la santé des consommateurs en améliorant la qualité sanitaire des denrées alimentaires, de la production primaire à la consommation**, (iii) **réduire les pertes aux stades de la production, de la transformation, de la distribution et/ou de la consommation**, (iv) **faciliter les échanges internationaux de produits agricoles et agro-alimentaires.**

Nous avons axé notre travail sur la recherche de stratégies pour réduire la présence de pathogènes dans des filières de production et de transformation alimentaire en conditions tropicales comme : (i) **l'application d'extraits de plantes antimicrobiens (huiles essentielles) en substitution des antibiotiques en aquaculture (Madagascar)**, (ii) **le traitement acide seul ou combiné à la vapeur pour décontaminer les peaux de volaille (la Réunion),** (iii) **l'application des bonnes pratiques d'hygiène à Madagascar pour maîtriser les dangers sanitaires** (bactéries pathogènes, mycotoxines) de différentes filières (agriculture périurbaine, épices, restauration de rue, élevage de porc). Ceci pour aider les

instances locales (Comité du Codex Alimentarius, Bureau des normes, Agence de sécurité sanitaire des aliments) à améliorer le système de contrôle des aliments.

Ci-dessous sont présentés nos axes de recherche principaux et les questions de recherche posées.

L'axe 1 concerne la recherche d'alternatives basées sur les extraits de plantes en substitution des antibiotiques en aquaculture. Les essais ont été menés dans un élevage de crevettes Bio à Madagascar.

Les huiles essentielles peuvent-elles constituer une alternative aux antibiotiques en aquaculture ?

- Quelles sont les compositions chimiques des extraits de plantes et leurs propriétés antimicrobiennes ? Quelles sont les variabilités intra spécifique et interspécifique chez *Cinnamosma* spp., une plante endémique malgache ? Les activités antimicrobiennes sont-elles comparables entre les huiles complexes et leurs composés majoritaires purs ?
- Quels sont les effets des huiles essentielles de *Cinnamosma fragrans* sur les concentrations bactériennes des larves de crevettes et de l'eau d'élevage (bactéries totales et populations de *Vibrio* spp.) ? Quels sont les effets sur les taux de survie des larves pendant le cycle larvaire (18j) ? L'effet de ces huiles essentielles est-il comparable à celui de l'antibiotique conventionnel ? Y a-t-il besoin d'un émulsifiant pour utiliser l'huile en conditions aquacoles et quel est son effet sur les activités des huiles ?

L'axe 2 porte sur l'application de traitements combinant la vapeur et l'acide lactique pour décontaminer des pièces de volailles inoculées avec des souches de *Listeria innocua* et *Salmonella enterica* Enteritidis. Ce travail é été réalisé dans le cadre d'une coopération avec un abattoir à la Réunion (Crête d'Or).

L'acide lactique seul, ou combiné à la chaleur, constitue-t-il un procédé innovant pour réduire la concentration de pathogènes sur les peaux de volailles ?

- Quels sont les effets de l'acide lactique utilisé seul ou combiné à la vapeur d'eau, pour réduire les concentrations de *Listeria innocua* (prise comme modèle de *Listeria monocytogenes*) et *Salmonella* Enteritidis sur des peaux de

volaille ? Quels sont les effets immédiats et les effets après conservation (4°C 7 jours) ?

- Quelles sont les conditions de traitement qui permettent d'allier, décontamination bactérienne et faisabilité industrielle, sans altérer les caractéristiques sensorielles des produits ?

Le 3e axe est spécifique au système de contrôle des denrées alimentaires dans les pays du sud. Il porte sur l'identification des dangers sanitaires et alimente une réflexion sur l'application de l'HACCP et des Bonnes Pratiques d'Hygiène (BPH), dans un pays où l'absence d'un système efficace de collecte et d'analyse de données le long de la chaîne alimentaire est reconnue comme une lacune majeure.

Quels sont les niveaux de contaminations des denrées alimentaires à Madagascar, de la production primaire à la consommation ou l'export?

- Quelles sont les concentrations de germes pathogènes, de germes indicateurs de l'hygiène ou des mycotoxines dans différentes denrées alimentaires : agriculture périurbaine (cresson, salade, feuilles de manioc pilées), plats consommés dans les ménages, restauration de rue (gargotes), épices destinées à l'export (vanille, girofle), viande de porc (abattoir et boucherie) ?
- Quelles sont les forces, les faiblesses, les opportunités et les menaces du système de contrôle national des denrées alimentaires pour l'application du système HACCP à Madagascar ?
- Quelles sont les recommandations pour améliorer la prévention des risques sanitaires des aliments dans le contexte local : élaboration des guides de Bonnes Pratiques d'Hygiène ?

2 - Expérience professionnelle dans le secteur privé :

Cette expérience professionnelle correspond à la période située entre le doctorat (1993) et l'entrée au Cirad (2002).

J'ai travaillé comme Consultante en Qualité (1994-1997 : Jordanie, Inde) puis comme Responsable Qualité chez des producteurs et exportateurs de crevettes (Gelpêche : 1999-2001).

J'ai travaillé dans plusieurs pays pour sélectionner des sites de production, agréer des usines et accompagner la mise en place du système HACCP pour l'export des produits de la mer en Europe (microbiologie, HACCP, normes européennes des produits et des locaux, traitement et conditionnement des produits). Je suis intervenue principalement à Madagascar où Gelpêche avait des installations de pêche et d'aquaculture, mais également en Inde, Iran, Nigéria, Maroc, Guyane, Brésil, Iles Canaries, Cameroun. J'ai expérimenté divers contextes de production en milieu tropical et apporté mon expertise pour améliorer la qualité des produits de la ferme à l'export (hygiène, analyses microbiologiques, HACCP, formation). J'ai été ainsi confrontée aux réalités de terrain et j'ai pu identifier les barrières majeures à la mise en place des bonnes pratiques d'hygiène et de l'HACCP, dans différents pays et contextes de production.

J'ai par exemple vécu l'embargo européen sur les crevettes à Madagascar et en Inde, en raison de la présence de *Vibrio* spp. et de résidus d'antibiotiques dans ces produits. La levée de l'embargo a été soumise à la mise aux normes des établissements (conception des locaux, bonnes pratiques d'hygiène, HACCP) et à la réalisation d'une analyse de risques pour justifier des moyens de maîtrise mis en œuvre par les opérateurs. L'UE a dans ce domaine mandaté les autorités compétentes dans ces pays respectifs pour assister les professionnels. En tant qu'interlocutrice avec les autorités officielles, j'ai connu les problèmes de sécurité sanitaire des denrées et les difficultés liées à leur maîtrise dans les pays du sud.

De nombreuses espèces du genre *Vibrio* spp. qui causent des mortalités importantes en aquaculture sont des germes autochtones de l'environnement marin. Les producteurs ont alors recours à l'usage des antibiotiques qu'ils utilisent en prophylaxie et en thérapie. Cependant, l'utilisation de molécules à large spectre et à des doses abusives, sans connaissance *a priori* des germes incriminés et de leurs antibiogrammes ont conduit à l'émergence de souches bactériennes résistantes dans l'environnement aquacole.

En intégrant le Cirad en 2002, j'ai développé des projets sur ce thème car l'aquaculture est un secteur en pleine croissance, surtout dans les pays du sud, et qui utilise massivement les antibiotiques. Cette intensification a des impacts négatifs sur l'environnement et sur la santé aussi bien animale qu'humaine (résistance aux antibiotiques des bactéries pathogènes pour les animaux ou pour l'homme).

3 - Substitution des antibiotiques en aquaculture

3.1. - Résistance des bactéries aux antibiotiques en aquaculture

L'aquaculture progresse plus rapidement que tous les autres secteurs de production alimentaire d'origine animale, avec un taux moyen annuel de 8,8 % depuis 1970, contre seulement 1,2 pour les pêches de capture et 2,8 pour les systèmes terrestres de production animale. Il est attendu que l'aquaculture atteigne une contribution à hauteur de 50% des approvisionnements de produits de la mer en 2015 (SOFIA, 2009). Pour ce secteur, les maladies infectieuses représentent un risque majeur et sont à l'origine de pertes économiques considérables. Pour prévenir ou traiter ces maladies, les aquaculteurs utilisent largement les antibiotiques. Or leur utilisation massive a entraîné l'apparition et la sélection de souches bactériennes résistantes à un, voire plusieurs antibiotiques. L'occurrence de ces souches pathogènes résistantes est une menace, non seulement en tant que source de maladies, mais aussi parce que leurs gènes de résistance peuvent facilement se propager à d'autres microorganismes pathogènes. Ceci constitue un danger pour la santé aussi bien animale qu'humaine (les gènes de résistance peuvent être transférés entre les bactéries de niches écologiques différentes). Des mesures ont donc été adoptées, aux niveaux national et international, pour réduire, voire éliminer, les antibiotiques en aquaculture.

Ces mesures sont indispensables pour préserver l'efficacité des antibiotiques et réduire les coûts pour les fermiers, mais demeurent cependant insuffisantes pour lutter contre la résistance aux antibiotiques. La littérature a en effet montré que (i) une fois acquis, les gènes de résistance peuvent être maintenus dans l'environnement même en absence de l'antibiotique correspondant, (ii) les impacts des pratiques d'élevage s'étendent en dehors de l'environnement de la ferme individuelle, (iii) en réponse à la pression exercée par les antibiotiques, les bactéries optimisent leur système de résistance envers plusieurs agents chimiques pour survivre (multirésistance), (iv) la résistance est souvent portée par des éléments génétiques mobiles facilement transférables entre bactéries.

La résistance à un antimicrobien est la capacité de certaines espèces de micro-organismes à survivre, voire à se multiplier, en sa présence à une concentration qui est habituellement suffisante pour inhiber ou tuer les bactéries de la même espèce. Les bactéries possèdent un mécanisme qui leur permet de résister à une concentration minimale inhibitrice (CMI) supérieure à la souche originelle ou sauvage. La résistance multiple ou multi-résistance est la résistance d'une souche bactérienne à plusieurs classes d'antibiotiques.

Voici quelques exemples de mécanismes de résistance aux antimicrobiens (Acar and Röstel, 2001) :

- Inactivation enzymatique de l'antimicrobien (modification chimique, hydrolyse).
- Réduction de l'accumulation intracellulaire de l'antimicrobien (absorption réduite par une baisse de la perméabilité membranaire, comme chez certaines bactéries à Gram négatif, systèmes de transport actif (efflux actif) protéiniques codés par des gènes de résistance : ces systèmes transportent en dehors de la cellule les principes actifs).
- Modification ou protection de la structure bactérienne cible.
- Enzymes de détoxication.

La résistance d'une bactérie à un antimicrobien a toujours une base génétique. Elle peut être spontanée (naturelle, inhérente, caractère stable) dans le cas de l'absence d'affinité entre l'agent antimicrobien et sa cible cellulaire, ou l'incapacité de l'agent à pénétrer la cellule, ou l'absence de cible dans la cellule. La résistance peut aussi être acquise, soit par mutation aléatoire, soit par l'acquisition de gènes codant pour un ou des mécanismes de résistance. Le transfert peut se faire par conjugaison, transformation ou transduction. La conjugaison permet le transfert direct d'ADN (plasmides, transposons) entre deux cellules bactériennes. La transformation correspond à l'intégration dans le chromosome bactérien de matériel génétique extérieur à la cellule. La transduction fait intervenir des virus bactériophages qui peuvent transmettre des morceaux d'ADN bactérien d'une cellule bactérienne à l'autre.
Or la résistance par mutation chromosomique n'est pas la plus fréquente et ne concerne pas un grand nombre d'antibiotiques. Seuls sont affectés les antibiotiques pour lesquels on observe une haute fréquence de mutation (rifampicine, acide fusidique, quinolones et phosphomycine).

Ces mécanismes de mobilité participent à la dissémination horizontale des gènes de résistance entre les cellules et confèrent le phénotype de multirésistance aux bactéries. Plusieurs résistances chez les bactéries de poisson sont portées par des éléments transférables comme les plasmides, les transposons et les intégrons (Adams *et al.*, 1998; Barlow *et al.*, 2004; Rosser and Young, 1999; Sandvang *et al.*, 1997; Yoo *et al.*, 2003), comme les résistances aux tétracyclines, chloramphenicol, sulphonamide, trimethoprime ou aux ß-lactames (Barlow *et al.*, 2004; Chopra and Roberts, 2001; Rosser and Young, 1999; Schmidt *et al.*, 2001; Sorum and L'Abee-Lund, 2002). Des intégrons portant des gènes de résistance aux antibiotiques ont été détectés dans plusieurs bactéries à Gram négatif comme *Escherichia coli*, *Proteus* sp., *Aeromonas* sp., *Morganella morganii*, *Shewanella* sp.

La résistance à divers antibiotiques a été largement décrite dans la littérature dans des bactéries isolées d'environnements aquacoles (eaux, sédiments, poisson) (Tableau 1).

La résistance de souches des genres *Vibrio* and *Aeromonas* a été plus élevée dans des écloseries de crevettes que dans les bassins de culture de *Penaeus monodon*, ce qui suggère davantage l'emploi des antibiotiques en écloserie plutôt qu'en ferme (Vaseeharan *et al.*, 2005). Dans plusieurs études, les niveaux de résistance bactérienne ont été corrélés avec les types d'antibiotiques administrés dans les élevages (Guardabassi *et al.*, 2000 ; McPhearson *et al.*, 1991 ; Spanggaard *et al.*, 1993 ; Tendencia and Leobert, 2002).

Tableau 1: Bactéries résistantes aux antibiotiques isolées d'environnements aquacoles.

Multiple antibiotic resistance patterns (a-b) Individual antibiotic resistance (a,b)	Bacterial species	Aquaculture Origin	Reference
Amx- Am- Ctx-C- Ffc- Te- Otc--E-Fx-Sxt*.	Gram negative bacteria	Salmon farms/Chile	(Miranda and Zemelman, 2002)
Otc-Ak-Am-Cb-Cf. Otc-Ak-Am-C-Ge-Net-Nf-Pef.	*Vibrio* spp.	Penaeid shrimps/ Mexico	(Molina-Aja *et al.*, 2002)
Am-C-Na-Ge-Te-Nf-To.	*Salmonella typhimurium*	Finfish/ India	(Ruiz *et al.*, 1999)
Am-C-E-Km-Sxt-Te.	*Aeromonas hydrophila*	Tilapia/ Malaysia	(Son *et al.*, 1997)
Am-C-Nf-Otc-Sxt-Na.	*Enterobacter gergoviae*	Catfish/ Vietnam	(Sarter *et al.*, 2007)
Oa-Sxt-Otc-Amx.	*Flavobacterium psychrophilum,* Aeromonads	Rainbow trout/ Denmark	(Schmidt *et al.*, 2000)
Am-Te-Sxt.	*Escherichia coli*	Seabob shrimps (Xiphopenaues kroyeri)/ Brazil	(Teophilo *et al.*, 2002)
Am, Amx, Ar, C, E, Enr, Ffc, Ge, Km, Oa, Otc, P, Sm, Sxt, Te.	*Photobacterium damselae subsp. piscicida*	Fish/ Japan	(Thyssen and Ollevier, 2001)
Am,Cht,Te,Km,Na, Nm.	*Vibrio* spp., *Aeromonas* spp.	Water *Penaeus monodon* hatcheries and ponds/ India	(Vaseeharan *et al.*, 2005)
Te-C-Km-Sa-Sm. C-Te-Oa-Sm.	*Escherichia coli*	Fish/ Korea	(Yoo *et al.*, 2003)

*Ak amikacin, Am ampicillin, Amx amoxicillin, Ar flumequine, Cb carbenicillin, Cf cephalotin, Ctx Cefotaxine, C chloramphenicol, Cht Chlortetracycline Enr enrofloxacin, E erythromycin, Ffc florfenicol, Fx Furazolidone, Ge gentamicin, Km kanamycin, Nm Neomycin, Net netilmicin, Nf nitrofurantoin, Oa oxolinic acid, Otc oxytetracycline, Pef pefloxacine, P penicillin, Sa sulphonamide, Sm streptomycin, Sxt trimethoprim-sulfamethoxazole, Sxt cotrimoxazole, Te tetracycline, To tobramycin

Les agents pathogènes portés par les produits aquacoles peuvent contaminer l'homme via la chaîne alimentaire ou bien par contact direct. La résistance des pathogènes humains est une menace en termes de santé publique car elle compromet l'efficacité des traitements. En fait,

plusieurs études ont décrit le transfert des gènes de résistance aux antibiotiques entre des bactéries entériques ou des souches cliniques et la microflore de poisson (Andersen and Sandaa, 1994 ; Furushita *et al.*, 2003 ; Goni-Urriza *et al.*, 2000 ; Rhodes *et al.*, 2000). Kruse et Sorum (1994) ont montré que la conjugaison et le transfert de plamides R peuvent avoir lieu entre des bactéries d'origines humaine, animale et halieutique appartenant à des niches écologiques différentes, et cela même en absence d'antibiotiques. Ainsi, des transferts sont possibles entre des espèces *Aeromonas* spp. et *E. coli*, entre des souches aquacoles et humaines provenant d'origines géographiques distinctes. En 1991, une épidémie de *Vibrio cholerae* 01 s'est propagée en Amérique Latine; la souche incriminée en Amérique Latine était sensible à 12 agents antimicrobiens, mais la souche retrouvée en Equateur est devenue multirésistante. L'épidémie de choléra en Equateur a débuté au sein de personnes travaillant dans les fermes de crevettes. Le phénotype multirésistant a été retrouvé chez des vibrions non cholériques qui étaient pathogènes pour les crevettes. Les auteurs pensent que les résistances observées chez *V. cholerae* 01 auraient été transférées à partir d'autres vibrions (Weber *et al.*, 1994).

L'étude que nous avons menée au Vietnam dans le cadre d'une Action Thématique Programmée (ATP financement propre du Cirad en 2002-2005) a montré des niveaux de résistance élevés à 6 antibiotiques majeurs dans des fermes de poissons chats situées dans le delta du Mékong (Fig1-2) (Sarter *et al.*, 2007). La majorité des isolats bactériens prélevés aléatoirement dans les 3 fermes étaient multirésistants (Tableau 2).
Les bactéries à Gram négatif oxidase positive (*Vibrio* sp.) ont été identifiées par galerie API 20NE ; les bacilles à Gram négatif oxidase négative (*Enterobacteriaceae*) par API 20E. Les souches de *Bacillus* spp. par galerie API 50CHB; *Micrococcus* spp. et *Stahylococcus* spp. par galeries APISTAPH (Gram+). Pour les Gram-négatifs, les tests biochimiques ont été réalisés selon les tests préconisés par l'Institut Pasteur (Le Minor and Richard, 1993 ; Richard and Kiredjian, 1995). Certaines souches ont été confirmées par l'Institut Pasteur de Lille (France).

Figure 1: Pourcentage de résistance des isolats Gram négatifs issus de fermes de poisson chat (CF : catfish farm) (n=92). CF1: 29 isolats, CF2: 40 isolats, CF3: 23 isolats. Les antibiogrammes ont été réalisés pour les 92 isolats contre 6 antibiotiques: oxytetracycline (OTC/30IU), chloramphenicol (C/30µg), trimethoprim-sulphamethoxazole (SXT/1.25µg-23.75µg), nitrofurantoin (FT/300µg), nalidixic acid (NA/30µg), ampicillin (AM/10IU).

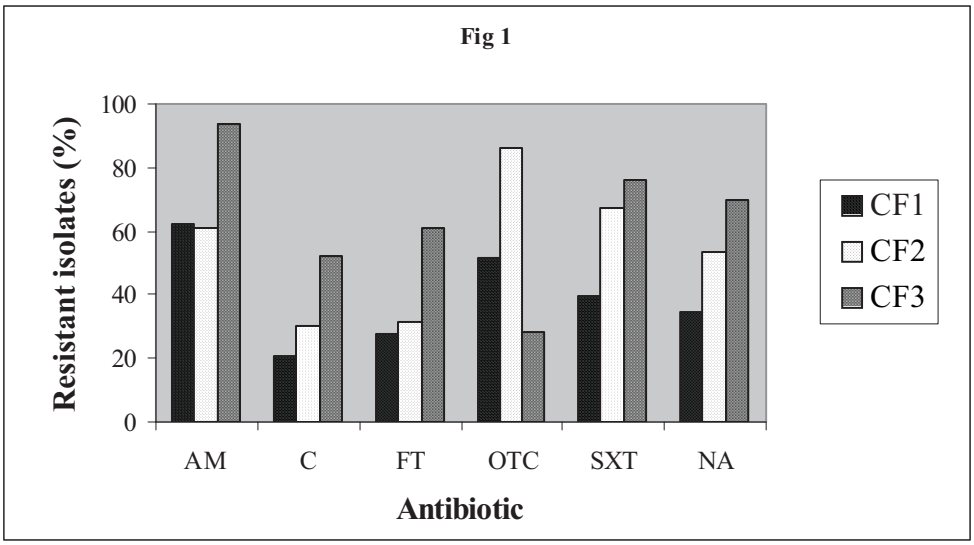


Figure 2: Multirésistance des isolats à Gram négatif sélectionnés arbitrairement des fermes d'élevage de poisson chat (CF1-CF3, n=92). CF: catfish farm, n: nombre des isolats.

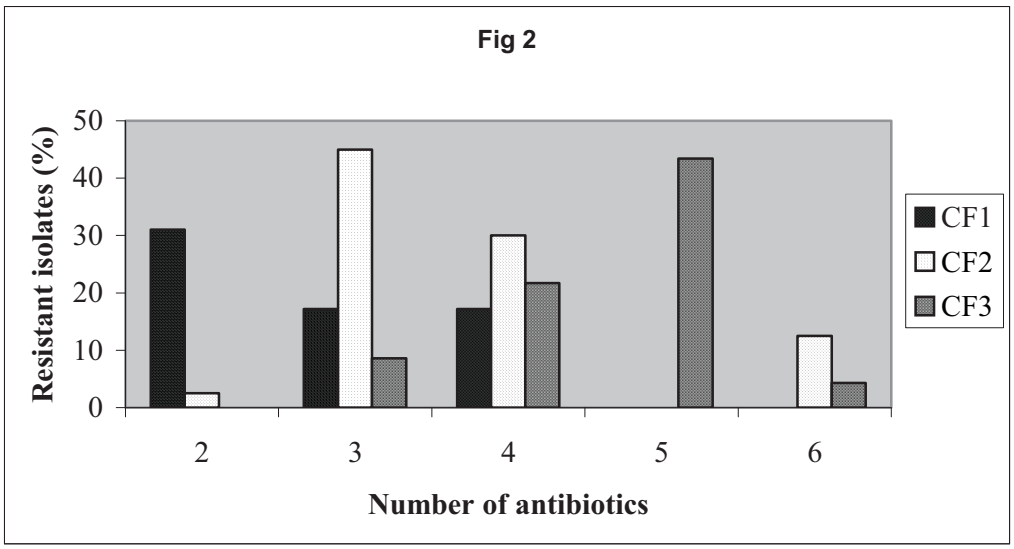


Tableau 2 : Profils de résistance aux antibiotiques chez 73 isolats bactériens multirésistants issus de fermes de poisson chat (Sarter *et al.*, 2007).

Profil de résistance	Espèces bactériennes	Nombre des isolats	% des isolats par profil
OTC, SXT	*Escherichia hermanii*	2	6,8
	Escherichia coli	3	
OTC, SXT, NA	*Escherichia hermanii*	1	15,1
	Xenorhabdus luminescens	9	
	Enterobacter agglomerans	1	
AM, OTC, SXT, NA	*Pseudomonas pseudomallei*	6	17,8
	Vibrio metschnikovii	2	
	Escherichia hermanii	3	
	Vibrio parahaemolyticus	1	
	Pseudomonas cepacia	1	
AM, FT, OTC	*Vibrio metschnikovii*	5	9,6
	Proteus vulgaris	1	
	Pragia fontium	1	
AM, C, OTC, SXT	*Escherichia coli*	5	6,8
AM, C, FT, OTC, SXT, NA	*Enterobacter gergoviae*	5	8,2
	Serratia plymuthica	1	
AM, C, FT, SXT, NA	*Pseudomonas cepacia*	10	13,7
Total		57	78,1

AM: ampicillin, C: chloramphenicol, FT: nitrofurantoin, NA: nalidixic acid
OTC: oxytetracycline, SXT: trimethoprim-sulphamethoxazole

L'analyse de la sensibilité de 123 isolats bactériens issus de l'eau et des sédiments de différentes fermes d'élevage (poisson chat, tilapia, carpe commune et gouramy) dans 5 provinces du fleuve Mékong (Vietnam) ont montré que 90% des isolats étaient résistants à la tétracycline, 76% à l'ampicilline, 100% au chloramphenicole, 65% au nitrofurantoine et 89% au trimethoprim-sulfamethoxazole (Phuong *et al.*, 2005). D'autres études ont montré l'augmentation des fréquences de résistance vis-à-vis de certains agents qui n'étaient pas ceux utilisés par les fermes (Austin, 1985). Cependant, la corrélation entre la résistance et la concentration des antibiotiques n'est pas toujours évidente parce que des fréquences de résistance élevées ont été observées dans des eaux n'ayant pas été en contact récent avec les agents antimicrobiens en question. Une incidence élevée oxytetracycline-resistant a ainsi été rapportée chez les populations d'aéromonades (69%) et de flavobactéries (72%), bien que cet antibiotique ait été peu utilisé en aquaculture au Danemark durant les 5 années précédant l'étude (Schmidt *et al.*, 2000). Aussi, une incidence inhabituellement élevée d'oxytetracycline-résistants (72%), trimethoprim-sulfadiazine résistants (44%) et

d'aéromonades multirésistants (50%) a été observée à l'entrée d'eau d'une des fermes à l'essai, ce point d'entrée étant proche de l'effluent d'une autre ferme. Il semblerait qu'une fois acquis, les gènes de résistance peuvent être maintenus au sein de la population bactérienne, même en absence de l'antibiotique (Chiew *et al.*, 1998). Ces résultats montrent l'importance de l'impact environnemental de la résistance aux antibiotiques en aquaculture.

Dans ce contexte, il devient nécessaire de développer des traitements alternatifs dans les pays producteurs afin d'assurer une aquaculture durable et responsable. Les forêts malgaches sont riches en espèces productrices d'huiles essentielles dont certaines plantes, déjà utilisées dans la médecine traditionnelle, seraient des candidates intéressantes pour remplacer les antibiotiques conventionnels car elles possèdent des propriétés antimicrobiennes. C'est le cas de *Cinnamosma fragrans*, arbre des forêts occidentales malgaches.

3.2. - Activités antimicrobiennes des huiles essentielles de *Cinnamosma fragrans*

A Madagascar, j'ai coordonné un Pôle d'Excellence Régional, projet financé pour 3 ans par l'AUF (2008-2011, 100000 euros). Ce Pôle scientifique régional a associé les Universités de Madagascar (Département de Biochimie Fondamentale et Appliquée ainsi que l'Ecole Supérieure des Sciences Agronomiques), des Comores et de la Réunion, et le Cirad à Madagascar. Ce pôle a formé 4 doctorants et une quinzaine de DEA.

Titre : **Valorisation des ressources de la biodiversité végétale de Madagascar et des Comores pour la sécurité des aliments : identification des plantes, criblage et caractérisation des molécules actives.**

La biodiversité malgache comporte 12000 espèces de plantes dont 85% sont endémiques. Malgré le nombre croissant d'études sur cette biodiversité végétale, nombreuses sont les espèces qui n'ont pas encore fait l'objet d'études approfondies pour la caractérisation de leur composition chimique et de leurs propriétés antimicrobiennes. Plusieurs de ces plantes sont cependant décrites par la pharmacopée locale et sont utilisées empiriquement pour soigner des pathologies infectieuses.

Madagascar possède des écosystèmes particuliers parmi les plus riches du monde avec un taux d'endémicité exceptionnel de près de 80%. *Cinnamosma fragrans* est une plante endémique de la famille des Cannellaceae. Le genre *Cinnamosma* sp. comprend trois espèces d'arbustes ou d'arbres : *C. fragrans,* distribué dans les parties nord, nord-ouest et centrales

ouest de Madagascar ; *C. macrocarpa et C. madagascariensis* qui sont localisés dans l'est, sud-est et dans la partie du sud de cette île. Cette plante présente des vertus curatives très vastes, et est utilisée en médecine traditionnelle contre les maladies de l'appareil respiratoire, les parasitoses intestinales, les maladies du foie, la syphilis (Pernet and Meyer, 1957) et la malaria (Milijaona *et al.*, 2003). Les fruits et les feuilles sont très aromatiques et produisent des huiles essentielles (Perrier de la Bâthie, 1954).

À notre connaissance, peu de littérature existe sur la composition chimique des huiles essentielles de *Cinnamosma* spp. et encore moins sur ses propriétés biologiques. La comparaison des résultats chimiques met en évidence une grande variabilité. En effet, une teneur élevée en linalool est remarquée dans l'huile essentielle produite par Schulte *et al.* (Schulte *et al.*, 1972), alors que le 1,8 cinéole et le sabinène dominent dans les échantillons décrits par Tucker *et al.* (Tucker *et al.*, 2008). La composition chimique des huiles essentielles de feuilles de *Cinnamosma fragrans* collectées dans deux régions ont montré deux compositions chimiques distinctes selon qu'ils contiennent majoritairement du linalool ou du 1,8 cinéole (Randrianarivelo *et al.*, 2009).

Les activités antimicrobiennes de 2 échantillons de ces huiles essentielles (B8 riche en linalool et B143 riche en 1.8 cinéole) ont été analysées *in vitro* vis-à-vis de différentes bactéries (*Vibrio harveyi, V. fisheri, V. penaeicidae, V. alginolyticus, V. anguillarium, V. splendidus, Micrococcus luteus, E. coli, Salmonella* Typhimurium*, Staphylococcus aureus, Bacillus subtilis*). Elles se sont avérées plus actives envers les espèces du genre *Vibrio*, spécifiques à l'aquaculture (Randrianarivelo *et al.*, 2009).

Ces résultats nous ont encouragés à tester ces huiles essentielles de *C. frangrans* dans l'écloserie d'une ferme d'élevage de crevettes Bio (*Penaeus monodon*) à Madagascar (LGA : Les Gambas de l'Ankarana). Les essais ont été conduits au niveau de l'écloserie pour évaluer les effets de l'huile sur les microflores (flore hétérotrophique totale, population de *Vibrio* sp.) des larves et de l'eau d'élevage, ainsi que le taux de survie des larves. Nous avons également testé les effets d'un émulsifiant (Tween 80) pour optimiser les conditions d'utilisation de l'huile dans un milieu aquacole.

Les valeurs des CMI variaient de 0,18 à 5,88 mg / ml, pour l'échantillon B8 (95.8% linalool) et de 0,37 à 11,8 mg / ml pour B143 (71,6% 1,8-cinéole) (Tableaux 3-4). Parmi les bactéries à Gram négatif, les souches les plus résistantes à l'extrait B8 ont été *V. alginolyticus* et

V.vulnificus. *V. parahaemolyticus* et *V. splendidus* ont quant à elles montré une plus grande résistance à l'échantillon B143.

En général, les échantillons de *C. fragrans* ont présenté, contre toutes les bactéries à Gram positif, des valeurs de CMI similaires à celles obtenues pour leurs composants majeurs purs respectifs, linalool et 1,8-cinéole. Les CMI de B143 ont été plus faibles contre toutes les souches à Gram négatif testées par rapport au composé pur, le1,8-cinéole. B8 a montré des valeurs de CMI plus élevées que le linalool contre *S.* Typhimurium et *V. alginolyticus*, et des CMI semblables à celles du linalool contre les autres souches de bactéries Gram négative.

Pour les souches isolées de l'écloserie les Bacillaceae ont été plus sensibles que les Vibrionaceae. Dans la plupart des cas, les valeurs CMB (concentration minimum bactéricide) pour B8 et B143 ont été équivalentes aux valeurs CMI (effet bactéricide)

Les tests de toxicité des huiles B8 et B143 aux stades Nauplii (jour 0), Zoé (jour 2), Mysis (jour 7) et Post larves (jour11) ont montré que la valeur moyenne de la DL50 était significativement inférieure pour B8 (863 mg/ml) par rapport à B143 (885 mg/ml) ($p < 0,05$ Fisher LSD).

Tableau 3: Activité antimicrobienne des extraits B8 et B143 de *Cinnamosma fragrans* originaires de Tsaramandroso (altitude) et de Mariarano (littoral) respectivement; ainsi que les activités antimicrobiennes du linalool et du 1.8-cinéole purs.

Souches de référence (ATCC)	Linalool		1.8-cinéole		B8 Tsaramandroso (95,8% linalool)		B143 Mariarano (71,63% 1.8-cinéole)	
	CMI mg/ml	CMB mg/ml	CMI mg/ml	CMB mg/ml	CMI mg/ml	CMB mg/ml	CMI mg/ml	CMB mg/ml
Gram-positif								
Micrococcus luteus	5,88a	11,75b	11,75b	11,75b	5,88a	5,88a	11,75b	11,75b
Bacillus subtilis	0,18a	0,18a	0,37b	0,73c	0,18a	0,18a	0,37b	0,73c
Staphylococcus aureus	0,18a	0,18a	0,37b	0,73c	0,18a	0,18a	0,37b	0,73c
Gram-négatif								
Salmonella tyhimurium	2,93a	5,88c	11,75b	11,75b	5,88c	5,88c	2,93a	5,88c
Escherichia coli	1,47a	1,47a	2,93b	2,93b	1,47a	2,93b	1,47a	1,47a
Vibrio fischeri	0,73a	1,47b	1,47b	1,47b	0,73a	0,73a	0,73a	0,73a
Vibrio anguillarum	1,47a	2,93b	2,93b	2,93b	1,47a	1,47a	1,47a	1,47a
Vibrio harveyi	1,47a	2,93b	2,93b	2,93b	1,47a	1,47a	1,47a	1,47a
Vibrio alginolyticus	1,47a	1,47a	5,88c	5,88c	2,93b	2,93b	1,47a	1,47a
Vibrio penaeicidae	0,18a	0,18a	2,93b	2,93b	2,93b	2,93b	0,18a	0,18a
Vibrio splendidus	5,88b	5,88b	2,93a	5,88b	2,93a	5,88b	5,88b	5,88b

CMI: Concentration Minimale Inhibitrice, CMB: Concentration Minimale Bactéricide
Les valeurs suivies de différentes lettres sur une même ligne sont significativement différentes selon le test de Fisher (p=0,05).

Tableau 4 : Concentration Minimale Inhibitrice (mg/ml) des échantillons B8 et B143 de *Cinnamosma fragrans* originaires de Tsaramandroso et de Mariarano respectivement, ainsi que le linalool et le 1.8-cinéole purs, contre des souches bactériennes isolées de la ferme d'élevage de crevettes (n=10).

Espèces bactériennes	**CMI (mg/ml)**			
	B8 (95,8% linalool)	**B143 (71,6% 1.8-cinéole)**	**Linalool**	**1.8-cinéole**
Gram positif				
Bacillus pumulus	0,18a	0,37b	0,18a	0,37b
Bacillus cereus	0,73a	1,47b	1,47b	1,47b
Bacillus subtilis	0,18a	0,37b	0,18a	0,37b
Micrococcus spp.	5,88a	5,88a	5,88a	11,75b
Gram négatif				
Vibrio hollisae	0,73a	0,73a	0,73a	2,93b
Vibrio alginolyticus	5,88c	2,93b	0,73a	2,93b
Vibrio parahaemolyticus	2,93a	5,88b	5,88b	2,93a
Vibrio vulnificus	5,88b	2,93a	5,88b	5,88b
Photobacterium damselae	2,93b	1,47a	1,47a	2,93b
Vibrio spp.	2,93a	1,47a	5,88b	1,47a

Les valeurs suivies de différentes lettres sur une même ligne sont significativement différentes selon le test de Fisher (p=0,05).

Tableau 5 : Identification des souches bactériennes isolées aléatoirement de l'eau (n=32) et des larves (n=48) des aquariums.

	Eau		Larves	
Espèces bactériennes	**Nb isolats (n=32)**	**(%)**	**Nb isolats (n=48)**	**(%)**
Bacillus pumulus	3	9	1	2
Bacillus cereus	2	6	1	2
Bacillus subtilis	2	6		
Micrococcus spp.	4	13		
total Gram positif		**34**		**4**
Vibrio hollisae	2	6	7	15
Vibrio alginolyticus	7	22	13	27
Vibrio parahaemolyticus	2	6	5	10
Vibrio vulnificus	3	9	6	13
Photobacterium damselae	2	6	4	8
Vibrio spp.	5	16	11	23
total Gram negatif		**66**		**96**

Figure 3: Evolution de la concentration bactérienne (cfu/ml) des larves de *P. monodon* obtenue sur marine agar pendant 18 jours de culture du témoin (sans huile essentielle, ni antibiotique), et des essais d'huiles essentielles de *C. fragrans* (B8 et B143) et de l'érythromycine (E). Les valeurs correspondent à la moyenne de 4 répétitions (n = 4 ± sd).

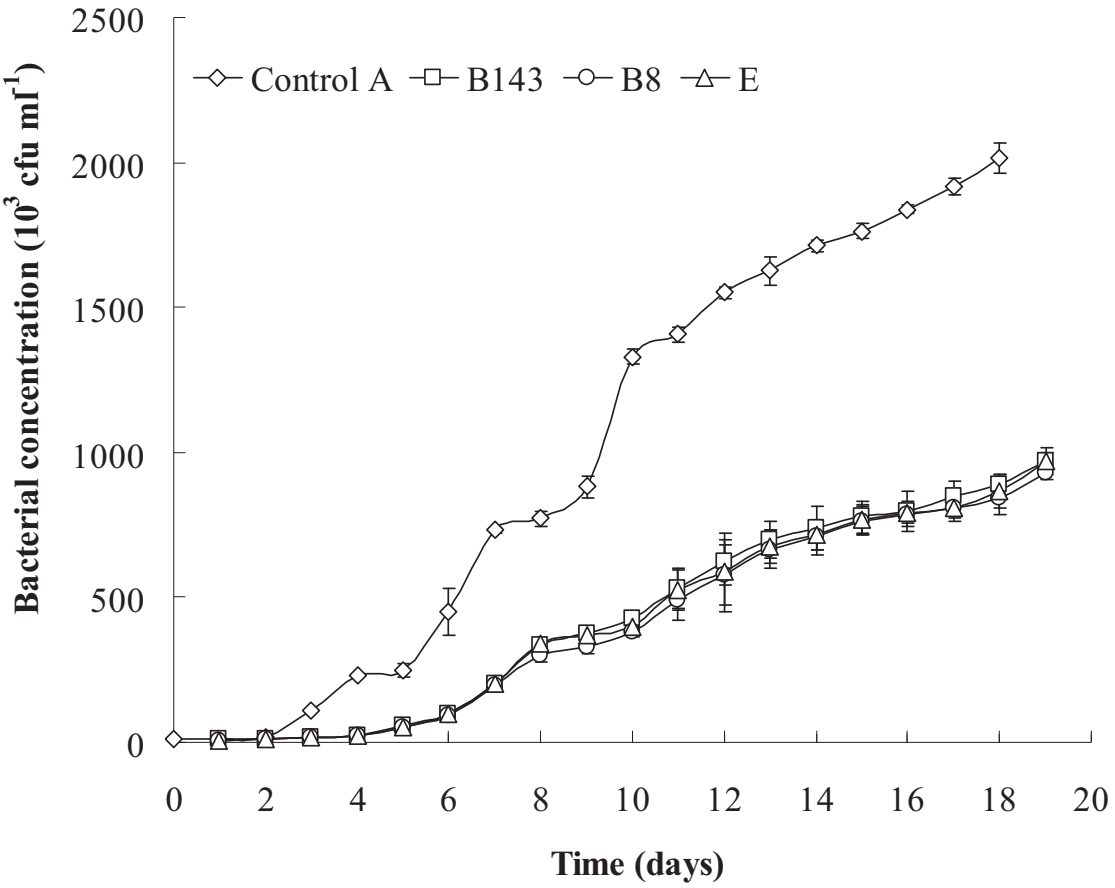
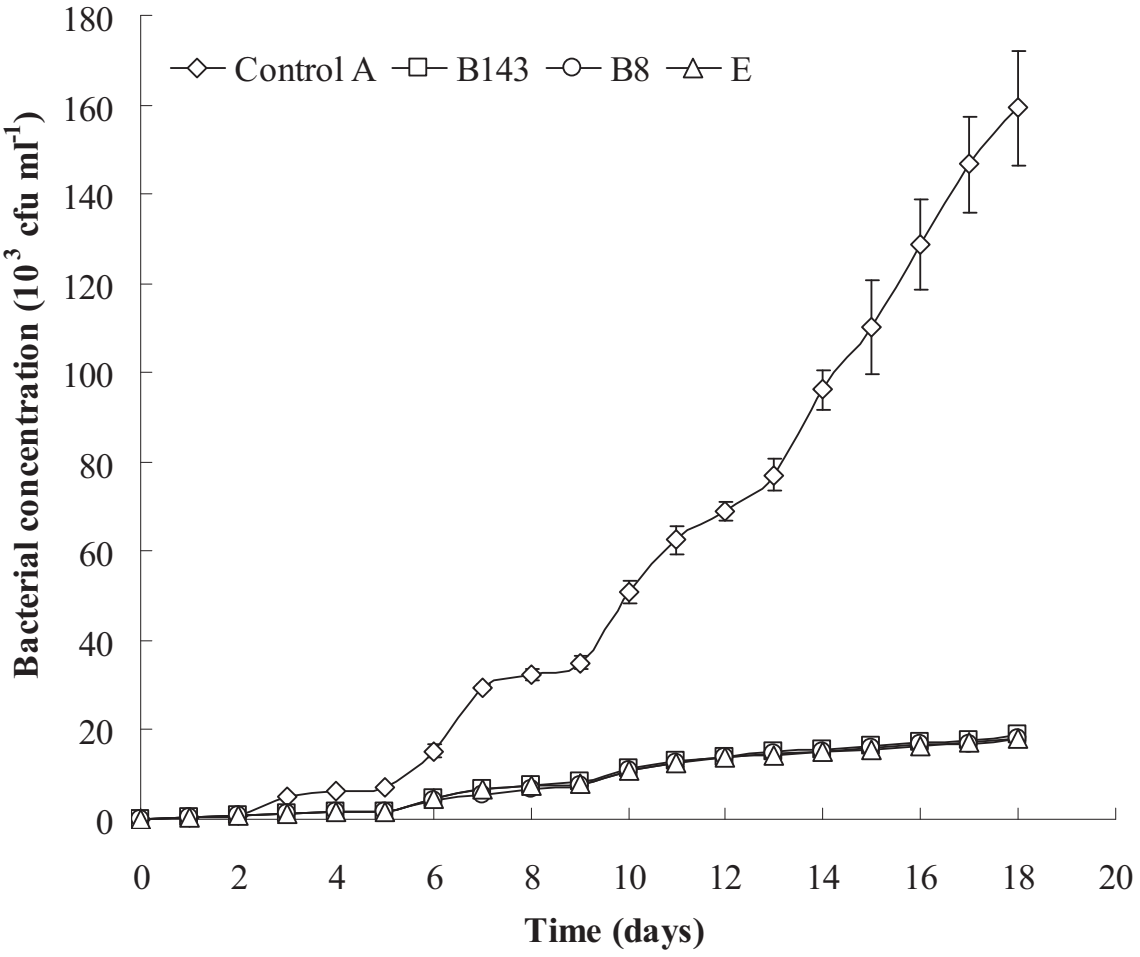


Figure 4: Evolution de la concentration de *Vibrio* spp. (cfu/ml) des larves de *P. monodon* pendant 18 jours de culture du témoin (sans huile essentielle, ni antibiotique), et des essais avec les huiles essentielles de *C. fragrans* (B8 et B143) et l'érythromycine (E). Les valeurs correspondent à la moyenne de 4 répétitions (n = 4 ± sd).



Parmi les souches isolées de l'écloserie, les espèces du genre *Vibrio* sp. représentent respectivement 66% des échantillons d'eau d'élevage (n= 32) et 96% des échantillons larvaires (n= 48) (Tableau 5).

Le dénombrement des bactéries totales sur Marine agar et le dénombrement de la population de *Vibrio* spp. sur TCBS jusqu'au stade PL 8 (jour 18) ont montré que les concentrations bactériennes des larves et celles de l'eau d'élevage traitée avec B8, B143 et l'antibiotique (E) ont été significativement plus faibles ($p < 0,05$) que celles du témoin (Fig. 3-4). En outre, il n'y a pas de différence significative ($p > 0,05$) entre les trois traitements (B8, B143 et E) (Randrianarivelo *et al.*, 2010 ; Sarter *et al.*, 2010a).

Pour chacun des traitements (B8, B143, E) et pour le témoin A, une corrélation significative a été observée entre les concentrations bactériennes des prélèvements larvaires et celles de l'eau d'élevage, avec des coefficients de corrélation importants ($r > 0,90$), avec une valeur $p < 0,05$ pour chacun des traitements ($p < 0,02$).

La survie des larves a été significativement plus élevée pour chaque traitement que pour le témoin (Fig. 5). Le test Kruskal-Wallis a montré que les effets de B8 et de B143 ont été significativement différents ($p<0,05$). Par contre, la différence entre les effets de l'antibiotique (E) et ceux de B8 n'est pas significative ($p> 0,05$). Pour les trois traitements, B8, B143 et E, la survie des larves de *P. monodon* à PL 8 a atteint respectivement 82,6%, 69,3% et 80,5%, tandis que celle du témoin n'a pas dépassé 15,1%.
Une corrélation significative a été observée entre les concentrations bactériennes des larves (bactéries totales sur Marine agar et vibrions sur TCBS) et le taux de survie à tous les stades larvaires. Par exemple la corrélation entre le taux de survie des larves à M3 (jour 9), PL1 (jour 11) et PL3 (jour13) et leurs concentrations bactériennes respectives sur TCBS a montré une corrélation négative significative ($r = -0,97$, $-0,97$ et $-0,98$ respectivement, $p <0,05$).

Concernant l'effet du Tween 80, l'utilisation de l'huile essentielle avec ou sans tween durant les 18 jours de culture a montré une p-value>0,05. Ces essais montrent que l'utilisation d'un émulsifiant (Tween 80) n'a pas eu d'effet significatif sur la concentration bactérienne des larves et leur taux de survie ($p> 0,05$) (Fig.6).

Figure 5 : Évolution du taux de survie (%) des larves de *P. monodon* pendant 18 jours de culture du témoin (sans huile essentielle, ni antibiotiques) et des essais des huiles essentielles de *C. fragrans* (B8 et B143) et de l'érythromycine (E). Les valeurs correspondent à la moyenne de 4 répétitions (n = 4 ± sd).

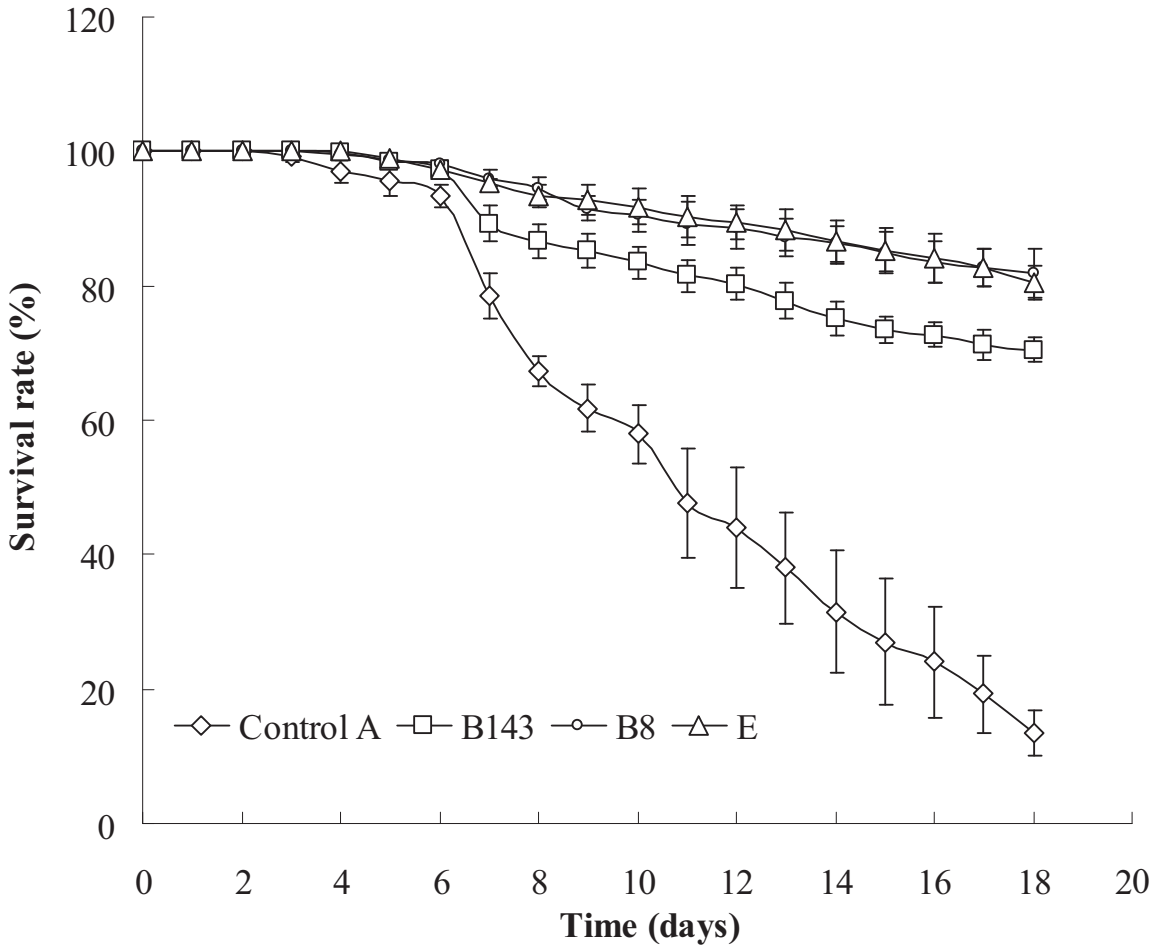
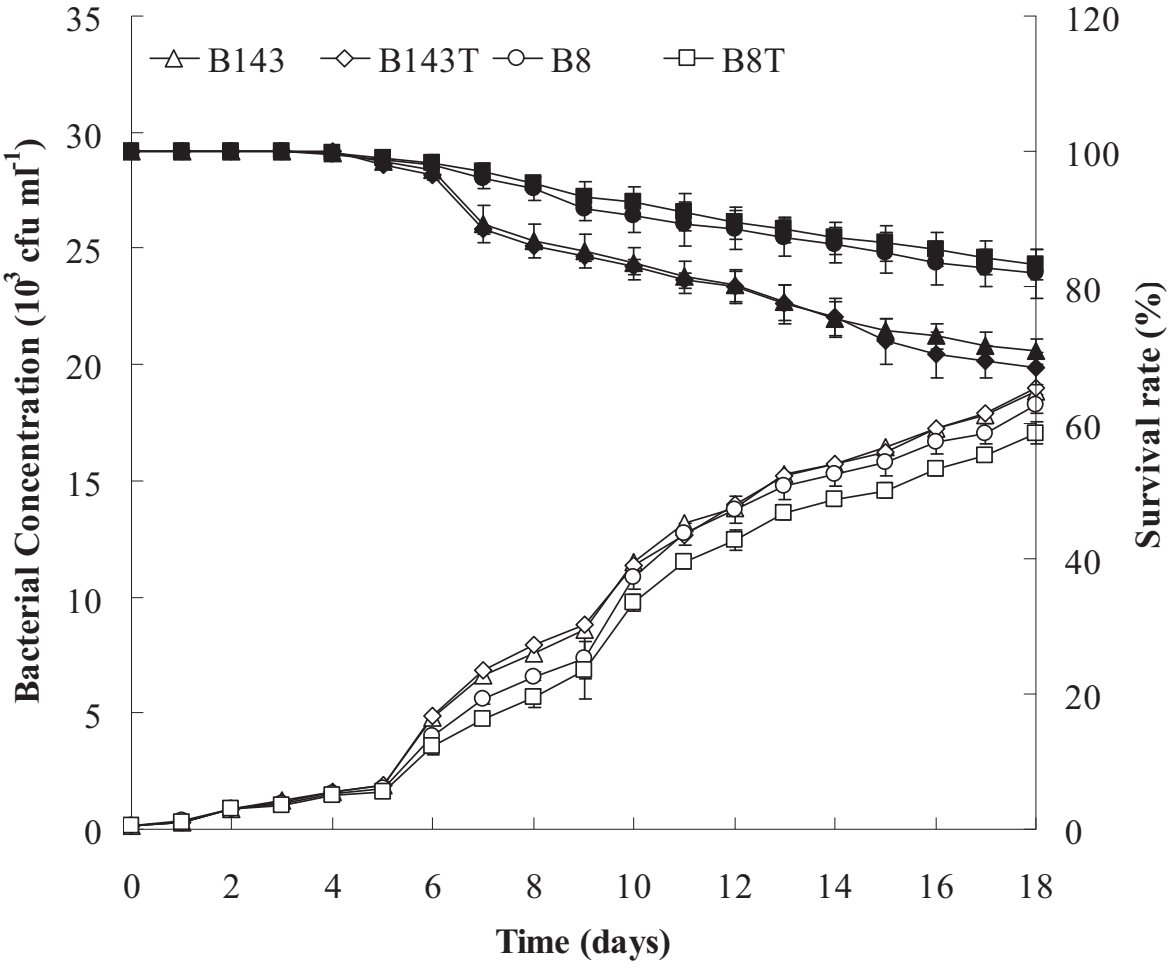


Figure 6 : Évolution de la concentration bactérienne (cfu/ml) et le taux de survie (%) des larves de *P. monodon*, pour les essais d'huiles essentielles de *C. fragrans* avec Tween 80 (B8T, B143T) et sans Tween 80 (B8, B143). Les symboles blancs correspondent à la concentration bactérienne (cfu/ml), et ceux noirs au taux de survie (%). Aucune différence significative n'est observée entre B8/B8T et B143/B143T ($p > 0,05$).



3.3. - Discussion des résultats

La famille des Vibrionaceae fait partie de la flore autochtone des organismes marins et constitue l'un des principaux groupes des environnements marins. Les espèces du genre *Vibrio* sp. isolées dans l'écloserie font partie de la diversité des populations retrouvées dans les élevages de *P. monodon* (Leaño *et al.*, 1998 ; Sung *et al.*, 1999). Les espèces des genres *Bacillus* sp., *Micrococcus* sp., *Staphylococcus* sp. ont été retrouvées dans les systèmes d'aquaculture (Nedoluha and Westhoff, 1997), et ont été également isolées sur milieu TCBS à

partir d'eau d'élevage d'Artemia (Kennedy *et al.*, 2006; López-Torres and Lizárraga-Partida, 2001). Les bactéries à Gram négatif sont le principal groupe isolé des larves et des juvéniles pénéides sains (Oxley *et al.*, 2002; Vandenberghe *et al.*, 1998).

Les plantes sont généralement considérées comme des sources riches en composés actifs (Citarasu *et al.*, 2002). Les résultats de la composition chimique de l'huile essentielle de *C. fragrans* sont proches de travaux décrivant d'autres huiles essentielles, dans lesquels les composants majeurs peuvent représenter jusqu'à 95% de l'huile essentielle, alors que les autres composants sont présents en faible quantité (Bauer *et al.*, 2001). Par exemple, les compositions chimiques des huiles essentielles de romarin et de sauge ont été également caractérisées par la présence prédominante de 1,8-cinéole, à hauteur de 88,9% et 78% respectivement (Daferera *et al.*, 2000). La littérature a montré que de nombreux facteurs, tels que l'origine géographique, les facteurs génétiques, le matériel végétal et la saison au cours de laquelle les plantes ont été récoltées peuvent être à l'origine des variations de la composition chimique d'une l'huile essentielle (Sivropoulou *et al.*, 1997).
Les principaux éléments constituant les huiles essentielles de *C. fragrans*, 1.8 -cinéole et le linalool sont bien connus pour leurs activités antibactériennes (Knobloch *et al.*, 1989 ; Viljoen *et al.*, 2003). D'autres constituants mineurs ont également été signalés pour leur activité antimicrobienne : p-cymène, α-pinène, β-pinène, limonène, α-terpinène, α-terpinolène, caryophyllène oxyde et camphène (Sökmen *et al.*, 2004). Nos résultats ont montré une activité antibactérienne plus élevée du linalool que du 1, 8-cineole contre les bactéries à Gram négatif, ce qui est en accord avec d'autres résultats (Faleiro *et al.*, 2003). Sibanda *et al.* (2004) qui ont testé l'activité antimicrobienne de l'huile essentielle de feuilles de *Heteropyxid dehniae* (le linalool étant le composant majeur 58,3%) contre différentes bactéries et champignons, ont trouvé que l'huile présentait des activités, soit similaires, soit différentes que le linalool pur selon le microorganisme testé. Faleiro *et al.* (2003) ont montré que *E. coli*, qui a été sensible au linalool pur, est résistante à un mélange contenant le linalool et le 1,8-cinéole (ratio 1:1). Les huiles essentielles sous forme de mélanges complexes, présentent des activités antimicrobiennes qui diffèrent de celles de leurs principaux composants testés seuls (Delaquis *et al.*, 2002). En fait, l'activité inhibitrice d'une huile essentielle traduit une interaction complexe entre ses différents constituants, qui peuvent produire des effets additifs, synergiques ou antagonistes, même pour ceux présents à des concentrations faibles (Xianfei *et al.*, 2007).

Dans la plupart des travaux publiés, les microorganismes à Gram positif semblent être plus sensibles aux huiles essentielles que ceux à Gram négatif (Dubber and Harder, 2008). Pour les souches isolées de la ferme, les espèces de la famille des Bacillaceae ont été plus sensibles que celles des Vibrionaceae. Les bactéries à Gram négatif possèdent une membrane externe qui joue un rôle de protection contre les éléments hydrophobes (Griffin *et al.*, 2001). La perméabilité des membranes des bactéries, la présence de pores protéiniques chez les bactéries Gram négatives et la distribution intracellulaire des constituants des huiles sont des éléments clés qui influent sur la diffusion et l'action de l'huile essentielle dans la cellule. Bien que les activités antimicrobiennes des huiles essentielles soient bien établies, leur mode d'action est encore mal compris (Baser *et al.*, 2006). Des effets cytotoxiques ont été observés *in vitro* dans des bactéries Gram positives et Gram négatives (Burt, 2004). Les terpènes ont été capables de rompre les membranes de *Bacillus thuringiensis* et *Bacillus cereus* entraînant la fuite des constituants intracellulaires (Andrews *et al.*, 1980 ; Ultee *et al.*, 1999). Le thymol et le carvacrol, deux des principaux composants de l'huile essentielle d'origan, ont endommagé l'intégrité de la membrane d'*Escherichia coli* et de *Salmonella* Typhimurium (Helander *et al.*, 1998). Cette huile essentielle a aussi désintégré la membrane de *Staphylococcus aureus* et *Pseudomonas aeruginosa*, et a perturbé l'homéostasie, le pH et l'équilibre des ions inorganiques (Knobloch *et al.*, 1989 ; Lambert *et al.*, 2001 ; Ultee *et al.*, 2002). Outre ces effets sur les membranes cellulaires, les composés actifs des huiles essentielles (comme les terpènes) peuvent avoir plusieurs cibles pour inhiber les croissances bactériennes (Tassou *et al.*, 2000). En raison de leur mode d'action touchant plusieurs cibles, il n'a pas été rapporté dans la littérature à ce jour de résistance particulière ou d'adaptation aux huiles essentielles (Bakkali *et al.*, 2008).

La corrélation négative observée entre la concentration bactérienne et la survie des larves dans les essais effectués avec les huiles essentielles B8, B143 et ceux avec l'antibiotique, a été en accord avec les résultats de Regunathan et Wesley (2004). L'inhibition de la croissance bactérienne a confirmé le potentiel des deux types de composition chimique de *C. fragrans* pour contrôler la charge bactérienne dans des conditions de culture larvaire *in vivo*. Ce potentiel peut être attribué à leurs effets antimicrobiens comme en témoigne leur faible CMI contre les différents isolats testés. Plusieurs études ont rapporté la possibilité d'utiliser des extraits de plantes comme alternative aux antibiotiques en aquaculture et en agriculture biologique en particulier. Ces extraits sont dans la plupart du temps mélangés directement à l'aliment. Abutbul *et al.* (2004) ont constaté une mortalité similaire dans le cas de tilapia

infecté par *Streptococcus iniae* pour des lots traités, soit avec un extrait de feuilles de *Rosmarinus officinalis*, soit avec l'oxytétracycline. Les juvéniles de *Penaeus indicus* nourris avec une alimentation enrichie d'un mélange de plantes terrestres donne de meilleures croissance et survie en inhibant la charge de *V. parahaemolyticus* (Immanuel *et al.*, 2004). L'administration d'artémies enrichies aux extraits de plantes *Withania somnifera* et *Mucuna pruriens* a montré un indice de qualité meilleur y compris pour ce qui concerne la survie des larves de *P. monodon* (Babu *et al.*, 2008). La protection obtenue par *Dendrilla nigra*, une éponge marine, a été signalée comme étant dû à son effet antibiotique contre *V. harveyi* et *V. alginolyticus*, plutôt qu'à son influence sur le système de défense des crevettes hôtes (Selvin and Lipton, 2004).

D'autres études en laboratoire ont conclu qu'il était inutile d'ajouter des émulsifiants pour l'utilisation des huiles essentielles (Dorman and Deans, 2000 ; Lambert *et al.*, 2001). Dans certains cas, les solvants et détergents ont montré un effet antagoniste qui diminue significativement l'activité antibactérienne des huiles essentielles (Remmal *et al.*, 1993). Le Tween 80 a montré divers effets sur les bactéries à des concentrations aussi faibles que 1%, 0,5% et 0,05% (Hood *et al.*, 2003). Afin de minimiser tout effet négatif de Tween 80, ces auteurs ont recommandé de conserver sa concentration inférieure à 0,05 % dans les tests utilisant les huiles essentielles.

Ces travaux se poursuivent pour étudier la variabilité géographique de *C. fragrans* et aussi étudier la seconde espèce du même genre présente à Madagascar : *Cinnamosma madagascariensis*. En ce qui concerne cette espèce, il n'existe pas de littérature disponible sur sa composition chimique et son pouvoir antibactérien. Il nous semble important de produire ces connaissances pour distinguer les caractéristiques de ces deux espèces du genre *Cinnamosma* sp. à Madagascar.

La première observation à partir de 50 échantillons analysés (30 échantillons de *C. fragrans* et 20 échantillons de *C. madagascariensis*) montre une similarité des composants majoritaires (1,8 cinéole et en linalool) des huiles essentielles des 2 espèces. 73,68 % de la composition chimique de l'huile essentielle de *C. fragrans* sont représentés par ce type de produits oxygénés monoterpéniques contre 50,94 % seulement pour *C. madagascariensis.* Par ailleurs, les teneurs de néral (0,10 % à 1,52 %) et géranial (0,00 à 5,35 %) sont plus faibles dans les huiles de *C. madagascariensis* que *C. fragrans* où ils peuvent atteindre 39% et 52% respectivement. Les huiles essentielles de *C. madagascariensis* sont aussi marquées par une forte teneur en produits oxygénés sesquiterpéniques avec un pourcentage relatif de 36,78 %

contre 9,66 % pour *C. fragrans.* Si le caryophyllène oxyde (7,14 % – 36,74 %) suivi de l'humulène oxyde (0,87 % – 9,48 %) et le β-eudésmol (0,00 % – 10,73 %) sont les principaux produits oxygénés sesquiterpéniques de l'huile de *C. madagascrariensis*, les échantillons de *C. fragrans* se démarquent par leur taux élevé en acide géranique (0,00 % – 55,70 %). Ces variations de la composition chimique auront-elles des effets sur les activités biologiques ? Le travail de thèse (doctorant Gaylor) qui est en cours essaie de répondre à cette question.

4 - Décontamination des peaux de volailles

4.1. - Effets des traitements sur la viabilité de *Listeria innocua* et *Salmonella* Enteritidis

Le niveau initial de contamination de la peau et de la surface des carcasses de volaille est déterminant pour la qualité sanitaire des produits transformés, en raison de la propagation des flores de surface vers l'intérieur du muscle (normalement stérile) et des contaminations croisées au cours des étapes de découpe et de transformation du produit. Malgré les efforts effectués dans les élevages, la peau des carcasses de volailles peut être contaminée, notamment par *Salmonella* spp., *Campylobacter* spp. et par des germes d'altération. A ces bactéries héritées de l'animal, s'ajoutent les contaminations par *Listeria monocytogenes* qui est susceptible de coloniser durablement les usines agroalimentaires, de former des biofilms et de contaminer ainsi des produits lors des étapes de transformation.

Les concentrations en acide lactique pour la décontamination de surface de viandes, présentées dans la littérature, sont habituellement faibles, de l'ordre de 1% à 2%. L'effet de décontamination, immédiatement après traitement, est bien moindre que dans le cas des traitements thermiques (réduction de 1 à 1,5 $\log_{10}$). Un phénomène de rémanence est souvent observé après traitement, ce qui conduit à un ralentissement de la croissance bactérienne (effet bactériostatique), voire à une inactivation supplémentaire (effet bactéricide) lors du stockage du produit. Aussi, l'étude d'un procédé combinant un traitement thermique et un traitement acide constituerait une alternative prometteuse pour concevoir des traitements doux qui n'altèrent pas les caractéristiques sensorielles des produits (aspect, goût).

Une thèse de l'université de la Réunion dont j'ai encadré les travaux en microbiologie a évalué l'efficacité des méthodes de décontamination de peaux de volailles par la vapeur d'eau (procédé Inra) et par l'acide lactique, utilisés seuls ou de manière combinée, dans des

conditions suffisamment douces pour ne pas détériorer les caractéristiques organoleptiques du produit frais. Ces traitements ont été testés en inoculant artificiellement *Listeria innocua*, dont la réponse aux traitements est similaire à celle de *Listeria monocytogenes*, et un germe pathogène, *Salmonella enterica* Serovar Enteritidis.

Globalement, l'efficacité du traitement par l'acide seul tend à augmenter avec la concentration en acide et la durée du temps de contact. La réduction de la population de *Listeria innocua* immédiatement après un traitement avec de l'acide lactique à 10%, peut atteindre 1,5 et 2,5 $\log_{10}$ ufc/cm^2 pour des durées respectives de contact de 1 min et 30 min (Lecompte *et al.*, 2008). Après le traitement, un effet bactéricide est observé en surface des produits (non rincés). La réduction supplémentaire de la population au bout de 7 jours de stockage peut être très importante. Elle est de 2,5 $\log_{10}$, pour une concentration en acide de 5% et un temps de contact d'une minute et de 6,0 $\log_{10}$ pour une concentration en acide de 10% et un temps de contact de 30 min. L'utilisation de l'acide lactique n'entraîne pas de modification des caractéristiques organoleptiques du produit pour des concentrations et des temps de contact allant jusqu'à 5%/1 min.
La teneur en acide lactique, dosée sur la totalité de la peau après 7 jours de stockage, n'augmente pas de manière significative sauf pour le traitement le plus drastique (concentration 10%, 30 min de contact : 700 µg/cm^2 contre 90 µg/cm^2 pour la peau non traitée).

L'effet d'un traitement combiné par la vapeur puis par l'acide a été étudié dans différentes conditions. Le traitement acide était appliqué après le traitement thermique, facilitant ainsi le refroidissement du produit pour simuler des conditions industrielles. Un effet de synergie peut être observé entre les traitements. Ainsi, une combinaison entre un traitement à la vapeur à 70°C/15 s, suivi d'un contact de la peau d'une minute avec une solution d'acide lactique à 5% a présenté, après 7 jours de conservation, une efficacité deux fois supérieure à chacun des traitements appliqué séparément. Les traitements combinés cumulent les avantages des deux traitements : une forte décontamination immédiate liée à l'effet thermique et un effet bactériostatique ou bactéricide durant le stockage lié à l'action acide (Tableau 6). Ainsi, les réductions bactériennes ont toujours été supérieures ou égales à celles de chaque traitement appliqué séparément, que ce soit immédiatement après traitement ou après 7 jours de conservation.

Tableau 6 : Concentrations en *Listeria innocua* et réductions décimales observées après des traitements thermiques et acides, seuls ou combinés (n=10 ± sd).

Traitement	Concentration bactérienne moyenne à la surface de la peau (log cfu/cm^2)		Réduction décimale moyenne (log cfu/cm2)	
	Jour 0	Jour 7	Jour 0	Jour 7
Témoin	6,65 ± 0,16^{a,b}	7,11 ± 0,33^{b}	-	-
Groupe A				
Thermique 98°C 10 s	2,33 ± 0,78^{c,d}	3,53 ± 0,97^{e,f}	4,32	3,57
Acide 10% 30 min	4,2 ± 0,57^{f,g}	<1,00h	2,45	>6,11
Acide 10% 1 min	5,18 ± 0,23^{g,i,j}	1,85 ± 0,83^{c,d,h,k}	1,47	5,25
Combiné: 98°C 10 s + Acide 10% 30 min	2,09 ± 0,8^{c,d,k}	<1,00	4,55	>6,11
Combiné: 98°C 10 s + Acide 10% 1 min	-	1,4 ± 0,73^{c,h,k}	-	5,71
Groupe B				
Thermique 70°C 1 min	1,2 ± 0,32^{h,k}	1,8 ± 0,56^{c,d,h,k}	5,44	5,3
Combiné: 70°C 1 min + Acide 10% 30 min	1,13 ± 0,25^{h,k}	<1,00	5,52	>6,11
Combiné: 70°C 1 min + Acide 10% 1 min	-	<1,00	-	>6,11
Groupe C				
Thermique 70°C 15 s	5,16 ± 0,42^{g,i,j}	5,38 ± 1,08$^{i.,j}$	1,49	1,72
Acide 5% 1 min	5,93 ± 0,14$^{a.,j}$	4,6 ± 0,72^{g,i}	0,71	2,54
Combiné: 70°C 15 s + Acide 5% 1 min	5,71 ± 0,62$^{a.,j}$	2,8 ± 0,87^{d,e}	0,93	4,3

Les concentrations bactériennes portant les mêmes lettres (a,b) ne sont pas significativement différentes.

Pour *Salmonella* Enteritidis (7 $\log_{10}$ cfu/cm^2), le traitement thermique seul (température 100°C, durée 8s) permet d'obtenir une réduction immédiate de plus de 4,5 log10 et aucune croissance des bactéries survivantes n'est observée durant le stockage. L'acide lactique seul (5% v/v 1 min) est moins efficace avec une réduction de 1,5 $\log_{10}$. Mais celle-ci augmente significativement durant le stockage pour atteindre 3,1 $\log_{10}$. Le traitement combiné est plus efficace : 6,2 $\log_{10}$ immédiatement et 6,6 $\log_{10}$ après stockage (Arnaud *et al.*, oral communication IUFoST, Cape Town 2010). Au cours du stockage, la charge bactérienne n'augmente pas de manière significative compte tenu de la variabilité des résultats. Le choc thermique n'augmente pas la résistance de *Salmonella* spp. à l'acide (Leyer and Johnson, 1993). Compte tenu de la variabilité des résultats, les niveaux de réduction ne sont pas significativement différents de ceux observés pour *Listeria innocua* dans des conditions de traitement similaires. Immédiatement après traitement thermique, la réduction bactérienne observée est proche des 2,91 $\log_{10}$ observées par McCann *et al.* (2006) sur de la peau de poulet inoculée avec *Salmonella* Typhimurium DT104 pour un traitement de 10 secondes avec de la vapeur à 100°C, la température de surface étant estimée à 87°C.

4.2. - Discussion des résultats

Plusieurs travaux scientifiques soulignent l'importance de l'effet de rémanence obtenu avec l'acide lactique. Ainsi, lors du traitement d'ailes de poulet par immersion dans un mélange d'acide lactique à 0,5% et de benzoate de sodium à 0,05%, après 8 jours de stockage à 4°C, les populations de *Salmonella* spp., *Campylobacter jejuni*, *Listeria monocytogenes* et *Escherichia coli* O157:H7 se sont avérées significativement plus faibles que sur des témoins non traités (réductions de l'ordre de 1,2 $\log_{10}$ pour *Listeria* spp. supérieures à 2,0 $\log_{10}$ pour *Salmonella* spp. et *Campylobacter* spp.) et la croissance des bactéries psychrotrophes a été retardée de manière significative (Hwang and Beuchat, 1994). Par ailleurs, sur des carcasses de poulet traitées à l'acide lactique à 1%, la réduction de la flore totale est supérieure après 6 jours de conservation à 4°C, atteignant 2,3 $\log_{10}$ pour un traitement par immersion (Okolocha and Ellerbroek, 2005). De même, sur du muscle de bœuf, après un traitement à l'acide lactique à 2% et durant 5 jours de conservation à 4°C, la réduction du nombre de *Salmonella* Typhimurium atteint 1,14 $\log_{10}$, et la réduction du nombre de *Listeria monocytogenes* 2,54 $\log_{10}$, alors que les réductions immédiates n'étaient que de 0,70 et 1,09 $\log_{10}$ respectivement. Dans le cas de *Salmonella* spp. il semblerait que l'acide lactique poursuive son activité bactéricide car il induit une réduction bactérienne significative par comparaison au témoin. Pour *Listeria monocytogenes* qui est psychrophile, la croissance bactérienne se poursuit au cours du stockage sur les témoins non traités. La croissance bactérienne est cependant plus faible sur les échantillons traités. Par conséquent, il s'agirait plutôt pour *Listeria monocytogenes* d'une activité bactériostatique de l'acide lactique (Gonzalez-Fandos and Dominguez 2006; Özdemir *et al.*, 2006).

Par ailleurs, l'effet de protection des traitements vis à vis d'éventuelles recontaminations est complexe à analyser. Deux phénomènes antagonistes entrent en jeu simultanément. La teneur résiduelle en produit de décontamination limite de manière générale la croissance des bactéries mais la disparition des flores de compétition du fait du traitement peut favoriser la croissance des pathogènes. Par exemple, Nissen *et al.* (2001) ont mené une étude sur des muscles de poulet et de bœuf et des peaux de porc préalablement traités par de l'acide lactique à 0,2M (1,8%), refroidies et inoculées respectivement avec *Salmonella* Enteritidis, *Yersinia enterocolitica* et *Escherichia coli* O157:H7 Après conservation à 10°C durant 5 jours à pression atmosphérique et 21 jours sous vide, la concentration en pathogènes augmente dans tous les cas au cours du temps et est généralement supérieure sur les pièces traitées par l'acide. La décontamination favoriserait ainsi la croissance de certains pathogènes dans le cas

d'une contamination ultérieure. Ceci est attribué à une diminution de l'effet de compétition en raison de la réduction de la flore totale suite au traitement par l'acide lactique. Inversement, Van Netten *et al.* (1998) ont étudié l'évolution de peaux de porc ayant subi une immersion dans des solutions d'acide lactique à 2% (traitement de décontamination) ou dans de l'eau seule (témoin). Ces peaux sont ensuite inoculées avec des bactéries pathogènes (*Campylobacter jejuni*, *Salmonella* Typhimurium, *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Yersinia enterocolitica* et *Listeria monocytogenes*) adaptées à l'acide lactique et aux basses températures par des techniques de culture spécifiques. Ils montrent que le nombre de bactéries évolue dans ce cas de manière semblable pour des peaux traitées et des témoins non traités. Ces résultats ont été observés à partir du premier jour de conservation et quelle que soit la température de stockage (4°C ou 12,5°C). Dans cette étude, le traitement de décontamination n'a donc pas d'effet sur les recontaminations ultérieures.

Des études menées *in vitro* montrent l'apparition de phénomène de tolérance à l'acide sur des bactéries cultivées dans un milieu contenant de l'acide lactique. La culture de *Listeria monocytogenes*, *Escherichia coli* O157:H7 et *Salmonella* Typhimurium dans des milieux liquides dont le pH est diminué grâce à de l'acide lactique permet d'obtenir des cellules plus résistantes à un choc acide. Les différences de réductions décimales entre bactéries adaptées et non adaptées peuvent dépasser 2,8 $\log_{10}$ pour *Salmonella* Typhimurium et *Listeria monocytogenes* (Koutsoumanis and Sofos, 2004 ; Samelis *et al.*, 2003). La résistance aux chocs acides semble dépendre également de la température. Ainsi en milieu liquide, *Listeria innocua* était moins résistante à des traitements acides appliqués à 4°C qu'à des traitements appliqués à 30°C (Koutsoumanis and Sofos, 2004).

Il faut ici distinguer la notion de choc, qui désigne l'exposition des bactéries à un stress important et de courte durée, de la notion de stress en général. Ainsi, une exposition de plusieurs jours à un pH de 6 est considérée comme un stress alors qu'une exposition de 1 minute à un pH de 2 est considérée comme un choc. La réponse commune des bactéries lorsqu'elles sont exposées à un stress se traduit par la production de protéines appelées facteurs sigma. Le rôle premier des facteurs sigma est de modifier l'affinité de l'ARN polymérase pour certains types de promoteurs, ce qui permet de moduler l'expression de certains gènes. Le facteur sigma principal contrôle ainsi l'expression d'un grand nombre de gènes indispensables à la vie de la cellule (Abee and Wouters, 1999). Les facteurs sigma alternatifs sont plus spécifiques, contrôlant un nombre de gènes plus faible. Chez *Listeria*

monocytogenes, ce facteur σ^B est produit en réponse à une grande variété de stress (osmolarité élevée, températures élevées ou faibles, éthanol, EDTA, froid) et lors de l'entrée en phase stationnaire qui entraîne un stress énergétique (Wemekamp-Kamphuis *et al.*, 2004). Chez *Escherichia coli*, c'est le facteur sigma alternatif σ^S, codé par le gène *rpoS* et contrôlant plus de 35 gènes différents, qui est exprimé lorsque la quantité de nutriments diminue et lors d'un stress général (Chung *et al.*, 2006). Ce même facteur σ^S est exprimé chez *Salmonella* Typhimurium en réponse aux stress osmotiques et acides et lors du passage en phase stationnaire (Bearson, 1996; McMeechan *et al.*, 2007).

Le système de tolérance acide, en revanche, peut fonctionner lorsque les bactéries sont dans un milieu minimal, soit en phase exponentielle de croissance, soit en phase stationnaire. Chez *Salmonella* Typhimurium, lors de l'exposition à un choc acide, plus de 50 protéines de choc acide (ASP, Acid Shock Protein) sont produites lorsque les cellules sont en phase exponentielle de croissance et 15 ASP ont été décrites en phase stationnaire (Abee and Wouters, 1999). Au moins 8 de ces protéines, indispensables pour la résistance à l'acide, sont contrôlées par le facteur sigma alternatif σ^S.

En plus de ces mécanismes de réponse spécifiques, des mécanismes de protection généraux sont mis en œuvre, en particulier pour réparer et stabiliser l'ADN (synthèse de RecA, DnaK) et assurer le repliement correct et le bon fonctionnement des protéines. Ainsi, lors d'un choc acide la synthèse des protéines chaperones DnaK, GroEL et du facteur sigma alternatif σ^B est augmentée chez *Listeria monocytogenes*, de la même manière que lors d'un choc thermique ou d'une augmentation de la teneur en sel du milieu (Cotter and Hill, 2003; Gandhi and Chikindas, 2007). Ces mécanismes de résistance sont responsables des phénomènes d'adaptation à l'acide fréquemment décrits dans la littérature. Par exemple, l'exposition de *Listeria monocytogenes* et de *Salmonella* Typhimurium durant 5 h à l'acide lactique (pH du milieu 5,5) multiplie par 10 environ leur résistance à un choc acide à pH 3 (Greenacre *et al.*, 2003). Les conditions de l'exposition au stress (durée, pH, type d'acide) modifient l'importance de la résistance à un choc acide ultérieur. Cette réponse dépend des pathogènes étudiés. Chez *Listeria monocytogenes*, *E. coli* O157:H7 et *Salmonella* Typhimurium, l'augmentation de la résistance aux traitements acides est observée après une adaptation à un stress à des pH de 5,0-6,0 ; 4,0-5,5 et 4,0-5,0 respectivement, l'adaptation étant maximale à pH 5,5 ; 5,0 et 4,5 respectivement (Koutsoumanis and Sofos, 2004). Ce phénomène d'adaptation à l'acide a été bien étudié par plusieurs auteurs (Greenacre *et al.*, 2003 ; Hill *et al.*, 1995; Samelis *et al.*, 2003).

Lors de l'exposition à un choc ou un stress thermique, les bactéries synthétisent également des protéines spécifiques (HSP, Heat Shock Proteins). Certaines HSP sont des molécules chaperon chargées d'assurer le repliement correct des protéines, comme par exemple GroEL, dont la structure est quasiment identique chez toutes les bactéries. D'autres HSP assurent la destruction des protéines non conformes. Certaines, comme DnaK et DnaJ facilitent la transcription de l'ADN et la traduction de l'ARN, en particulier en protégeant les ribosomes. D'autres HSP sont des protéases ATP-dépendantes comme la protéine ClpC de *Listeria monocytogenes* ou ClpP chez *Bacillus subtilis*, chargées de dégrader les protéines non conformes (Abee and Wouters, 1999). Chez *Escherichia coli* et *Bacillus subtilis*, plusieurs facteurs sigma alternatifs sont produits en cas d'exposition à un stress ou un choc thermique (σ^{32}, σ^{N}, σ^{B}). En cas de stress extrême (températures supérieures à 42°C), un facteur spécifique, σ^{E}, appartenant à une classe de facteurs sigma répondant à la dégradation de protéines du périplasme, contrôle l'expression d'une trentaine de gènes assurant la production d'un autre ensemble de protéines (Chung *et al.*, 2006). La réponse aux chocs thermiques est rapide. Chez *Listeria monocytogenes*, dans les 5 minutes après un choc thermique à 45°C la transcription du gène codant la protéine GroEL est multipliée par cinq.

L'exposition des bactéries à un stress thermique peut donc augmenter leur résistance à un choc thermique ultérieur. Ainsi, la résistance d'une culture de *Salmonella* Typhimurium face à un choc thermique à 52°C est multipliée par 4 lorsqu'elle a préalablement été soumise à un stress thermique à 48°C durant 30 minutes (Bunning, 1990). La durée de la protection acquise après un stress thermique est extrêmement variable selon les microorganismes et les conditions du stress (durée et température), et varie entre 1 et 10 heures (Bunning, 1990).

Chez *Listeria monocytogenes*, l'exposition à un stress acide à pH 5 à 6 durant 90 minutes n'apporte pas de résistance supplémentaire face à un choc thermique à 50°C (Koutsoumanis *et al.*, 2003). De même, sur des cellules de *Listeria monocytogenes,* en phase exponentielle de croissance, un stress thermique à 45°C durant 1 h n'augmente pas la résistance à un choc acide à pH 3,5 (Lou and Yousef, 1997). La résistance aux chocs est complexe et dépend également de l'intensité du choc final. Après exposition de cultures de *Listeria monocytogenes* à des combinaisons de stress acides, thermiques et osmotiques, la résistance est augmentée face à un choc thermique à 57°C, mais pas face à un choc thermique à 52°C ou 63°C (Skandamis *et al.*, 2008).

Cependant, il faut souligner que la plupart des résultats de la littérature présentés ci-dessus sont obtenus en milieu liquide (bouillon nutritif). Ces articles ne décrivent pas des traitements

à des températures plus élevées, de bactéries fixées sur une surface alimentaire, comme dans le cas de la décontamination des surfaces de viande et de volaille.

Sur des matrices alimentaires, McCann *et al.* (2006) ont obtenu des réductions de *Salmonella* Typhimurium DT104 de l'ordre de 3,65; 5,23; 6,15 et 2,64 $\log_{10}$ cfu/cm^2 après un traitement à la vapeur de 87°C /60s appliqué sur des surfaces de bœuf, de poulet, de peaux de poulet et de porc respectivement. Cependant ces auteurs ont montré qu'un traitement à la vapeur dépassant 10s donnait un aspect cuit au produit traité. Sur des carcasses de porc, les flores mésophiles et psychrophiles indigènes sont réduites de 0,7 et 1,3 $\log_{10}$ respectivement après un traitement par la vapeur (température de la vapeur de 90 à 95°C, durée du traitement estimée à 0,013 s) suivi d'une pulvérisation d'acide lactique à 2% (à 45°C). Après 5 jours de stockage à 4°C, ces réductions sont de 1,5 et 3 $\log_{10}$ respectivement (Pipek *et al.*, 2006). L'efficacité du traitement combiné est significativement plus importante que celle du traitement à la vapeur appliqué seul. Des résultats du même ordre ont été obtenus sur des carcasses de boeuf (Pipek *et al.*, 2005). De la même manière, sur du muscle de bœuf inoculé avec *Escherichia coli* et *Salmonella* Typhimurium et traité avec des solutions d'acide acétique à 1, 2 ou 3% appliquées à des températures de 25, 40, 55 ou 70°C, la concentration en acide a une influence prépondérante sur les réductions bactériennes entre 25 et 55°C. A 70°C, en revanche, l'augmentation de la concentration en acide n'accroit pas de manière significative les réductions bactériennes (Anderson and Marshall, 1989).

Il est important de souligner que les résultats décrit dans la littérature en matière de décontamination des surfaces de viande ou de volaille sont très variables. En effet, cela dépend de nombreux facteurs incluant les espèces pathogènes testées, la flore indigène présente sur l'aliment, la composition de l'aliment, la sévérité du traitement (type et concentration de l'acide, température, durée), l'activité de l'eau ou le pH de l'aliment. Nos recherches permettent ainsi de définir les conditions optimales de décontamination de la surface des carcasses de volaille en vue de proposer des traitements efficaces, assurant une réduction significative de la charge bactérienne des carcasses sans modifier leurs propriétés sensorielles. En effet, dans les pays industrialisés, le contrôle des conditions d'élevage et les bonnes pratiques d'hygiène peuvent s'avérer insuffisants pour éviter la transmission alimentaire des espèces pathogènes (zoonoses). C'est pourquoi, des traitements innovants sont fortement recherchés par les industriels pour garantir l'innocuité des produits transformés. Ce

travail a fait l'objet d'un transfert de technologie vers un abattoir à la Réunion (Crête d'or) partenaire de ce projet.

5 - Système de sécurité sanitaire des denrées alimentaires dans les pays en développement :

5.1. - Analyse du système de contrôle des aliments à Madagascar

A Madagascar, les maladies diarrhéiques représentent environ 18% du nombre total de morts, ce qui en fait la 3e cause de mortalité après les infections respiratoires (27%) et le paludisme (22%). Ces maladies représentent 22% de morts chez les enfants de moins de 5 ans, ce qui en fait la première cause de mortalité dans cette classe d'âge (WHO, 2008). Les aliments (incluant l'eau potable) ont été reconnus comme étant la source majeure véhiculant les maladies diarrhéiques (Motarjemi and Käferstein, 1999). En général, dans les pays en développement, l'incidence et les coûts relatifs aux maladies transmises par les aliments, sont plus élevés que dans les pays développés (Henson, 2003). Or, Madagascar fait partie des pays les plus pauvres de la planète avec un Produit Intérieur Brut d'environ 830 USD/personne; et 61% de la population vit en dessous du seuil de pauvreté (WHO, 2006). Dans ce contexte, il nous semble important de produire des connaissances sur la qualité des denrées produites à Madagascar, mais aussi d'analyser le système de contrôle des aliments pour mettre en évidence les besoins et les points faibles à améliorer.

La responsabilité d'une nourriture saine et nutritive incombe à l'ensemble des parties prenantes dans le système alimentaire, c'est-à-dire à tous ceux qui produisent, transforment et commercialisent des aliments. Il est donc nécessaire de mettre l'accent sur l'élimination préalable des dangers sanitaires tout au long de la filière alimentaire, de la ferme ou de la mer à la table. Cette démarche vise à compléter l'approche traditionnelle de la gestion de la sécurité sanitaire des aliments qui est fondée sur la réglementation et le contrôle des produits finis. Au sein des filières alimentaires, la sécurité sanitaire des aliments doit englober les trois composantes fondamentales de l'analyse des risques : évaluation, gestion et communication. Dans ce processus d'analyse, il doit y avoir une séparation institutionnelle entre l'évaluation des risques à base scientifique et la gestion des risques. Des connaissances scientifiques solides et des systèmes efficaces de notification de l'incidence de maladies d'origine alimentaire sont nécessaires pour établir des priorités fondées sur les risques. Toutefois, si la recherche scientifique et la production de connaissances indépendantes constituent les

fondements d'une bonne évaluation des risques, il est important de noter que la gestion des risques, en elle-même, suppose un processus politique qui peut être déficient dans les pays du sud comme Madagascar.

L'HACCP est assimilé à une norme internationale de sécurité sanitaire des aliments par le Codex Alimentarius. De nombreux pays développés (Etats-Unis, UE) ont fait de son adoption une exigence réglementaire, principalement pour certaines denrées importées (viande, poisson). Ce phénomène a incité les pays en développement à mettre en place des règles du système HACCP dans leurs propres législations. Toutefois, cette adoption se limite bien souvent aux établissements qui produisent, transforment et manipulent des produits alimentaires destinés à l'exportation, et ne s'applique pas au marché local, comme c'est le cas à Madagascar. En effet, la mise en place d'un système HACCP est couteuse et de nombreux pays ne disposant que d'une expertise et de ressources humaines limitées, tendent à ne l'appliquer qu'aux secteurs les plus rentables économiquement.

La sécurité des aliments est donc le résultat de plusieurs facteurs : la législation doit fixer des exigences d'hygiène minimales ; des contrôles officiels doivent être mis en place afin de vérifier que les opérateurs se conforment à ces exigences ; ces opérateurs doivent élaborer et mettre en œuvre des programmes et des procédures de sécurité alimentaire fondés sur les principes HACCP. Au final, c'est bien une approche intégrée qui est nécessaire pour garantir la sûreté alimentaire du lieu de production primaire jusqu'à la mise sur le marché.

Nous avons mené une réflexion critique du système de sécurité des aliments à Madagascar afin d'identifier les forces et les faiblesses pour mettre en place le système HACCP et les Bonnes Pratiques d'Hygiène (BPH) (Sarter *et al.*, 2010b). En nous aidant de la littérature (Hathaway, 1999; Orriss and Whitehead, 2000; Spreij and Vapnek, 2007), nous avons mis en perspective les 4 actions principales suivantes pour renforcer le rôle du Gouvernement quant à l'application des BPH et de l'HACCP, à Madagascar:

A/ Mise en œuvre d'une politique nationale

- Elaborer les stratégies pour l'application des BPH et de l'HACCP dans les filières alimentaires, centraliser les informations, définir les freins et en identifier les causes, établir les priorités pour mettre en place les BPH et HACCP en fonction des types d'aliments, des infrastructures et des process

- Créer l'environnement pour promouvoir les BPH et l'HACCP en éliminant les contraintes et en promouvant des moyens incitatifs (d'un point de vue légal ou financier)
- Clarifier les rôles et les responsabilités des agences impliquées

B/ Compréhension scientifique du risque

- Développer une évaluation des risques pour des types de produits à risques et définir les objectifs de sécurité permettant d'établir des contrôles des dangers sanitaires fondés sur les principes de BPH et d'HACCP le long des filières alimentaires
- Appliquer les principes de l'analyse de risque pour toute prise de décision

C/ Législation et application des exigences réglementaires

- Fournir les lois décrivant les principes fondamentaux du contrôle des aliments
- Fournir la réglementation préconisant les exigences obligatoires et les objectifs à atteindre
- Définir les standards et les cahiers des charges que les opérateurs doivent satisfaire pour gérer les risques
- Etablir les sanctions en cas de non-conformité pour renforcer la mise en œuvre des standards

D/ Renforcement des autorités publiques

- Clarifier les responsabilités entre les différents services officiels impliqués ; établir un plan de communication et de coordination efficace entre ces services pour appliquer les principes généraux d'hygiène des aliments, les codes d'usage (préconisés par le Codex Alimentarius), ainsi que la législation nationale en vigueur en partenariat avec les professionnels du secteur alimentaire
- Apporter un appui en termes de sensibilisation et de formation aux entreprises et aux agents d'inspection
- Etablir la réglementation concernant les critères et les objectifs de performance pour les opérateurs du secteur alimentaire
- Conduire des inspections à l'improviste, valider les plans de BPH et HACCP, valider les guides industriels élaborés par les filières professionnelle

- Préparer des plans génériques pour assister les petites et moyennes entreprises dans l'élaboration de leurs plans BPH/HACCP internes. Assurer une communication efficace avec le secteur industriel pour développer ensemble des guides de bonnes pratiques utilisant des documents simplifiés (usage collectif)

Afin de faciliter l'évaluation des forces et des besoins pour aider les autorités malgaches à appliquer les BPH et l'HACCP dans le secteur alimentaire, une analyse SWOT (Strength, Weakness, Opportunity, Threat) est représentée dans le Tableau 7.

Les faiblesses apparentes de la situation à Madagascar en matière de sécurité sanitaire des aliments sont communes aux pays en développement. Les systèmes de production sont en général extrêmement divers et constitués de marchés informels et de petits producteurs inorganisés. Le secteur alimentaire se développe rapidement dans ces pays, mais avec peu d'appui technique pour l'introduction de nouvelles technologies et de pratiques satisfaisant les critères de qualité dans les petites et moyennes entreprises. Souvent, le secteur industriel de la transformation des produits alimentaires est sous-financé et fragmenté. Le pouvoir d'achat des consommateurs est trop faible pour que se crée une demande effective de produits de meilleure qualité. Dans ces pays, des cadres réglementaires incomplets ou obsolètes, un manque de ressources humaines et de moyens techniques (dont des laboratoires) efficaces pour le contrôle des aliments constituent une entrave à la mise en place de HACCP et des BPH (Panisello and Quantick, 2001). Ces faiblesses systémiques menacent la santé publique et risquent également de se traduire par un accès réduit aux marchés alimentaires mondiaux (embargo européen à Madagascar sur les produits d'origine animale depuis 1997, hormis les produits halieutiques, faute d'analyse de risque pour la mise en place d'un plan de surveillance documenté et efficace des maladies animales). De plus, les consommateurs des pays en développement sont en général préoccupés par l'accès aux aliments en quantités suffisantes et guère sensibilisés aux questions de sécurité sanitaire des aliments (Mortlock *et al.*, 1999).

Tableau 7 : Analyse des Forces, Faiblesses, Opportunités et Menaces liées à l'application des BPH et de l'HACCP dans le secteur agroalimentaire à Madagascar (Sarter *et al.*, 2010b).

	FORCES	**FAIBLESSES**
FACTEURS INTERNES	A/ Mise en oeuvre d'une politique nationale - La gestion de la sécurité des aliments est inscrite dans l'agenda politique (MAP, PNN, PNAN)	A/ Mise en œuvre d'une politique nationale - Aucune stratégie n'est définie pour l'application des BPH et HACCP. - Aucune incitation n'existe pour les industries alimentaires du marché local pour mettre en place les BPH et HACCP.
	B/ Compréhension scientifique du risque - Une agence du contrôle de la qualité et sécurité des aliments a été établie en 2006 - Existence de centres de recherche publics pour conduire des études - Le Plan d'Action National de Nutrition souligne la nécessité de conduire des recherches sur la qualité et sécurité des aliments	B/ Compréhension scientifique du risque - La gestion de la sécurité des aliments n'est pas basée sur une analyse de risques - Aucun support n'est apporté à la recherche (analyse de risques). - Manque de capacités pour l'analyse de risques dans les agences et les laboratoires officiels
	C/ Législation et application des exigences réglementaires - Projet de loi alimentaire en cours - Réglementation du secteur laitier en cours en partenariat avec les professionnels - Existence du Bureau des Normes de Madagascar pour établir les normes et standards nationaux - Participation à des initiatives internationales and régionales : Codex, Comesa, Sadc	C/ Législation et application des exigences réglementaires - La sécurité des aliments n'est pas basée sur la responsabilité des opérateurs professionnels - La législation est fragmentaire et dépassée - Faibles contrôle, application et sanctions pour évaluer la mise en conformité - Manque de standards, de spécifications, de directives, de guides pour les professionnels du secteur agroalimentaire
	D/ Renforcement des autorités publiques - Plusieurs agences impliquées dans la gestion de la sécurité des aliments et officiellement chargées de développer les BPH et HACCP auprès des opérateurs - Présence d'autorités compétentes ASH (export des produits de la mer), Services Vétérinaires (produits d'origine animale), - Existence du CNCA pour divulguer les principes du Codex et offrir une plateforme de discussion entre différentes institutions	D/ Renforcement des autorités publiques - Pas d'infrastructure en charge de l'application des BPH et HACCP - Confusion et duplication des responsabilités de plusieurs agences impliquées dans la gestion de la sécurité des aliments à des niveaux central et local - Manque d'expertise, de ressources, de budget et d'équipement des agences publiques

FACTEURS EXTERNES	**OPPORTUNITES**	**MENACES**
	- Opportunité pour obtenir une assistance financière et technique de la part des bailleurs internationaux pour améliorer les capacités - Présence de secteurs leaders comme le tourisme, l'export et la population expatriée (crevettes, litchis, fraises…) - Présence d'un laboratoire accrédité (LHAE) - Présence d'organismes de certification privée - Expérience dans la conduite de campagne de communication en santé publique (vaccination) - Existence de "Fokontany" qui peuvent intervenir au niveau du district	- Confiance limitée des gouvernements étrangers en termes de qualité et sécurité des aliments produits localement - Niveau d'éducation limité des parties prenantes et des consommateurs - Connaissances très limitées de l'hygiène des aliments chez les opérateurs et les consommateurs - Faible pouvoir d'achat des consommateurs n'encourage pas les opérateurs à investir dans des mesures de qualité plus couteuses (hygiène, système HACCP, assurance de la qualité)

ASH: Agence Sanitaire Halieutique; CNCA: Comité National du Codex Alimentarius; Comesa: Common Market for Eastern and Southern Africa; BPH: Bonnes Pratiques d'Hygiène; HACCP: Hazard Analysis and Critical Control Point; LHAE: Laboratoire d'Hygiène des Aliments et de l'Environnement de l'Institut Pasteur de Madagascar; MAP: Madagascar Action Plan; PNAN: Plan National d'Action pour la Nutrition; PNN: Politique Nationale de Nutrition; Sadc: Southern African Development Community.

5.2. - Maîtrise des dangers microbiologiques des denrées alimentaires

La qualité microbiologique ne peut être maîtrisée et garantie qu'à travers une approche intégrée se déclinant à tous les stades de la chaîne alimentaire, depuis la production primaire jusqu'à la consommation finale. Les microorganismes, qu'ils soient d'altération ou pathogènes, peuvent nuire à la qualité et à la sécurité des produits. Nous avons mené plusieurs études pour évaluer les conditions hygiéniques de production et identifier des dangers sanitaires de certaines denrées. Ceci afin de développer des stratégies visant à prévenir ou limiter la contamination des produits (guides de bonnes pratiques d'hygiène, démarche HACCP).

Nous avons ainsi mené des études (certaines sont en cours) pour analyser la qualité microbiologique (flore mésophile aérobie, *E. coli*, *Salmonella* spp., *Staphylococcus aureus*) de différentes denrées destinées au marché local ou à l'export :

- Analyses microbiologiques (*Salmonella* spp., *S. aureus*, *E. coli,* champignons) et des mycotoxines (Aflatoxine, Ochratoxine) de la vanille et du girofle aux

différents stades de production et de transformation (paysans, collecteurs, exportateurs), projets UE/ 2009-2010.

- Analyse microbiologiques (flore aérobie mésophile totale, *E. coli*, *S. aureus*, *Salmonella* spp.) des salades composées vendues dans les gargotes (article soumis en novembre dans Social Science & Medicine)
- Analyses microbiologiques de quelques légume-feuilles au niveau de la production (agriculture urbaine), des marchés de gros et dans les ménages d'Antananarivo (travail en cours)
- Détermination de la résistance aux antibiotiques des isolats de *E. coli* et *Salmonella* spp. de viande de porc au niveau des abattoirs et des boucheries. Identification des souches de *Salmonella* spp isolées (travail en cours).

Cas des épices :

Nous avons analysé la vanille et le girofle aux différents stades de production-distribution, incluant les producteurs (paysannat), les collecteurs et les exportateurs. Nous avons dénombré la flore aérobie mésophile, les levures et moisissures, *E. coli*, les staphylocoques pathogènes et recherché la présence de *Salmonella* spp. dans 129 échantillons de vanille et 50 échantillons de girofle prélevés aléatoirement.

La flore aérobie mésophile n'a pas directement une incidence sanitaire mais rend compte de la charge bactérienne globale. Ce critère est un indicateur des conditions d'hygiène générales de manipulation des produits. Il est inférieur à 5 log en moyenne pour le girofle et compris entre 6 et 7 log pour la vanille.

Concernant *Salmonella* spp (absence/25g), *E. coli* (<10 ufc.g) et *Staphylococcus aureus* (<100 ufc/g), les résultats sont satisfaisants pour tous les échantillons de vanille et de girofle.

Les concentrations en levures et moisissures sont également satisfaisantes (< 5log ufc/g).

Nous avons alors déterminé les étapes critiques devant être maîtrisées pour ne pas dépasser les seuils de tolérance.

Les mycotoxines (aflatoxine et ochratoxine) ont été recherchées dans 39 échantillons de vanille et 30 de girofle prélevés aléatoirement. Tous sont négatifs pour les deux mycotoxines.

Nous avons préconisé la mise en place de bonnes pratiques d'hygiène au cours du traitement et du stockage de ces produits pour limiter le risque de développement microbien et de synthèse des mycotoxines.

Nous avons préconisé l'élaboration de guides de bonnes pratiques qui constituent un outil précieux pouvant aider les exploitants à respecter les règles d'hygiène alimentaire à toutes les

étapes de la chaîne alimentaire et à appliquer les principes HACCP. Dans le cadre de deux projets financés par l'UE (programmes d'appui à la qualité des épices destinées à l'export en Europe), nous avons réalisé deux guides de bonnes pratiques d'hygiène en partenariat avec le Centre Technique Horticole de Tamatave pour la production et la transformation de la vanille et du girofle.

Cependant, la qualité de ces épices reste en général peu valorisée à l'export. En effet le girofle est en majorité exporté en grade de niveau 3 (dernière catégorie sur une échelle allant de 1 à 3) et vendu principalement en Indonésie pour la confection de cigarettes. En ce qui concerne la vanille, 80% de la production est destiné à l'extraction (vanille plus sèche nécessitant moins de soins exportée principalement aux Etats-Unis) et seulement 10 à 20% est classé en vanille noire dite de bouche (exportée en Europe). Cela montre que la qualité de ces produits pourrait encore être améliorée pour cibler des marchés plus rémunérateurs.

Cas des salades composées dans les gargotes :

A Madagascar, peu de données sont disponibles sur l'hygiène et la qualité des denrées vendues par les petits restaurants de rue (appelés communément gargotes). A Antananarivo, dans chaque quartier de la ville il y a des petits restaurants qui vendent à toutes heures des snacks (salades mixtes, beignets, nouilles…) et des repas chauds (riz avec sauce aux légumes et à la viande). Bien qu'il n'y ait aucune statistique officielle, nous estimons que ce phénomène concerne au moins plusieurs dizaines de milliers de consommateurs chaque jour.

Ces restaurants de rue ont généralement une infrastructure rudimentaire, avec peu ou pas d'accès à l'eau courante, aux toilettes, aux moyens de désinfection de l'eau, au réfrigérateur ou à la glace, ainsi qu'à l'évacuation correcte des déchets.

Nous avons choisi ce sujet car il n'a pas fait, à notre connaissance, l'objet d'une investigation approfondie à ce jour. Nous avons analysé la qualité microbiologique de la salade composée vendue par 30 restaurants auprès desquels, nous avons réalisé des observations ponctuelles des indicateurs d'hygiène comme la présence/absence de (1) réfrigérateur, (2) animaux dans l'environnement proche du restaurant, (3) vaisselle sale, (4) eaux usées, (5) protection de l'eau potable et (6) habits sales (Tableau 8). En notant ces observations (0 ou 1), nous avons établi un index d'hygiène pour chaque restaurant.

Les analyses des échantillons de salade composée (n=120) dans les trois quartiers testés d'Antananarivo sont de qualité insatisfaisante selon les critères microbiologiques correspondant à ce type de denrées (Gilbert *et al.*, 2000). La concentration d'*E. coli* est en effet supérieure au seuil limite de 10^2 cfu/g dans 83% des échantillons (Fig.7) et *Salmonella*

spp. a été détectée dans 10% des échantillons (n=60). Une corrélation positive a été retrouvée entre les concentrations en *E. coli* des salades et l'index d'hygiène des gargotes (test de Pearson, p=0,05).

Figure 7 : Concentrations bactériennes (log_{10} cfu/g) des échantillons de salade mixte dans les restaurants de rue d'Antananarivo (n=60±SD). ACC : Aerobic Colony Count

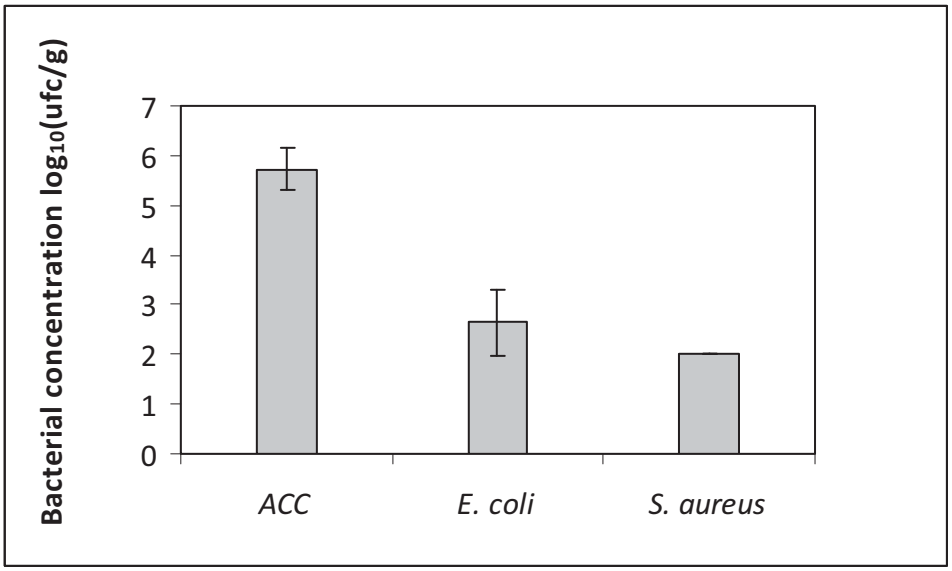


Tableau 8 : Observations spot des indicateurs d'hygiène dans les restaurants de rue d'Antananarivo (n=30)

Observations ponctuelles	**Pourcentage (%)** (n=30)
Absence de réfrigerateur	62
Présence d'animaux (chien, chat, volaille…)	60
Présence de vaisselle sale	93
Présence d'eaux usées	93
Eau potable non couverte	53
Habits sales du vendeur	90

Cependant l'hygiène n'a pas seulement une dimension microbiologique mais aussi socio-culturelle. Aussi nous avons collaboré avec un chercheur sociologue pour réaliser cette étude. Il s'agissait pour nous de comprendre les pratiques liées à l'hygiène de ces restaurateurs et de chercher comment motiver un changement de comportement pour améliorer l'hygiène (article soumis dans Social Science & Medicine, novembre 2010). Cet aspect social est d'une

importance cruciale car avant de vouloir changer les pratiques et les conceptions des gens, il faut connaître les pratiques existantes et les motivations qui les sous-tendent.

Discussion globale :

Il est essentiel de compléter l'approche actuelle en matière de réglementation et de contrôle des systèmes de sécurité sanitaire des aliments par des mesures préventives visant à réduire la contamination des aliments à la source. Cela nécessite l'adoption de bonnes pratiques d'hygiène au niveau de la production alimentaire, du traitement après récolte, de la transformation et de la manipulation. Les BPH permettent de réduire les risques de contamination microbiologique, physique et chimique dans la filière alimentaire. Dans certains cas, le danger ne peut simplement pas être supprimé, par exemple, les dangers liés à des bactéries opportunistes présentes naturellement dans le milieu. Les entreprises agroalimentaires malgaches, pour la majorité de petite et moyenne tailles, n'ont pas les ressources financières et techniques pour développer des démarches qualité individuelles (HACCP, BPH). Nous pensons qu'il est nécessaire de s'appuyer sur des démarches collectives pour améliorer les connaissances des opérateurs et pour renforcer les capacités des autorités locales à contrôler les denrées, sur la base de guides de BPH validés par l'administration et les opérateurs. L'élaboration de ces guides doit se fonder sur la démarche HACCP et sur des analyses objectives de la qualité des denrées. Ceci afin de déterminer les étapes critiques des process qui nécessitent une surveillance et des contrôles continus. La collaboration entre les services officiels et les professionnels doit être encouragée dans ce sens (FAO/WHO, 2005).

6 - Encadrement d'étudiants en Master et Doctorants

Doctorants :

- Jean-Yves Lecompte, Université de la Réunion, 2004-2008
  - Décontamination de peaux de volailles par utilisation d'une solution d'acide lactique et de vapeur d'eau. Effet des traitements seuls ou combinés.
  - Directeurs de thèse : Antoine Collignan (Cirad), Alain Kondjoyan (Inra)
  - Encadrant en microbiologie : Samira Sarter (Cirad, UMR Qualisud)
  - Thèse soutenue le 1.07.2008

- Roger Randrianarivelo, Université d'Antananarivo, 2004-2010
    - Étude de l'activité antimicrobienne de l'huile essentielle extraite d'une plante de Madagascar *Cinnamosma fragrans* : Application en crevetticulture en vue de substituer l'antibiotique.
    - Directeur de thèse : Marson Raherimandimby (Université d'Antananarivo)
    - Co-directeur : Pascal Danthu (Cirad, UR Forêt et Biodiversité)
    - Co-directeur et encadrant principal : Samira Sarter (Cirad, UMR Qualisud)
    - Thèse soutenue le 25.06.10 (Mention très honorables avec les félicitations du jury)
- Gaylor Razafimamonjison, Université d'Antananarivo, 2008-2011
    - Variabilités de la composition chimique des huiles essentielles des deux espèces endémiques de Madagascar (*Cinnamosma fragrans* et *Cinnamosma madagascariensis*) et leurs activités antimicrobiennes. Application dans l'aquaculture de crevette
    - Directeur de thèse : Panja Ramanoelina (Université d'Antananarivo)
    - Co-directeurs : Pascal Danthu (Cirad, UR Forêt et Biodiversité) et Samira Sarter (Cirad, UMR Qualisud)
- Eliane Razafinsalama, Université d'Antananarivo, 2008-2011
    - Caractérisation chimique et biologique de substances à propriétés antimicrobiennes issues de *Dilobeia thouarsii* une plante endemique malgache
    - Directeur de thèse : Victor Jeannoda (Université d'Antananarivo)
    - Co-directeur : Samira Sarter (Cirad, UMR Qualisud)

Masters :

Une vingtaine de DEA et Master 2 entre 2004-2010 sur les sujets suivants :

- Analyse de la résistance des bactéries en aquaculture de poisson en Asie (Kha Nguyen, Leopold, Isabelle Agui, Seyha, Stéphanie Jaubert) et dans la viande de porc à Madagascar (Franck, Tovo)
- Etude des propriétés antimicrobiennes d'extraits de plantes issus de la biodiversité végétale malgache (Mounidati, Mamihery, Sandra, Djanza, Hari Tiana, Jeannot)
- Analyse microbiologique des légumes (cresson, salade, ravitoto) au niveau des sites de production, des marchés et dans les ménages d'Antananarivo (Rova, Josette, Seheno, Tovo, Hiary, Zo)

- Traitement de décontamination de viande par Déshydratation, imprégnation par immersion (DII) (FZ. El Kaouti)

Ces étudiants sont d'origines diverses : Vietnam, Cambodge, Cameroun, France, Madagascar, Comores, Maroc.

7 - Enseignement

J'ai enseigné comme professeur consultant à l'Ensia Siarc à Montpellier (convention avec le Cirad) entre 2002 et 2006, une vingtaine d'heure/an sur la démarche qualité dans les filières de production de crevettes en région chaude et sur le système HACCP.
J'ai aussi enseigné au Vietnam, formation financée par l'AUF (Agence Universitaire de la Francophonie) pour les filières d'enseignement francophones (30 h en 2005 à la Faculté des Pêches de Nha Trang) : cours et travaux dirigés sur le système HACCP appliqué à l'aquaculture.
J'ai dispensé des cours à Madagascar, à la faculté de Pharmacie (5h/an) sur les pathogènes transmis par les aliments.
La Faculté des sciences d'Antananarivo à Madagascar (département de Biochimie Fondamentale et Appliquée où je suis accueillie) est en cours de mise en place de la réforme LMD. Il est envisagé que j'enseigne 15h/an dans le nouveau système qui rentrera en vigueur en 2011 dans la formation de Master sur Santé et Biodiversité.

8 - Conclusions et perspectives de recherche

Depuis mon entrée au Cirad en 2002, l'ensemble de mes activités de recherche m'a permis de contribuer à la production de connaissances nouvelles pour améliorer la qualité sanitaire des aliments dans le contexte des pays tropicaux. En poste à Madagascar depuis 2006, je me suis attachée à développer des projets de recherche pertinents pour mon pays d'accueil mais qui portent sur des sujets d'intérêt global et publiables dans des revues internationales. Avec des chercheurs malgaches et du Cirad, **nous avons démontré pour la première fois, la possibilité de substituer les antibiotiques en aquaculture par des huiles essentielles antimicrobiennes.** L'action des huiles essentielles de *Cinnamosma fragrans*, ciblant les populations de *Vibrio* spp. en aquaculture a permis d'obtenir en fin de cycle de croissance larvaire un taux de survie des larves similaire à celui obtenu avec l'antibiotique conventionnel et une réduction significative des concentrations de vibrions dans l'eau d'élevage et dans les

larves. Ces recherches, sur les principes actifs des extraits de plantes, nous ouvrent de nouvelles perspectives pour lutter contre le développement de résistances aux antibiotiques en aquaculture. J'envisage d'approfondir cet axe de recherche dans l'avenir en Asie. J'ai déjà soumis un projet à BioAsia 2010 (40000 euros) pour étudier les activités antimicrobiennes des extraits de plantes locales dans différents pays de la région (Malaisie, Vietnam, Cambodge). Le sud-est asiatique est un géant de l'industrie aquacole (poisson, crevettes) où l'utilisation massive des antibiotiques est reconnue comme un problème majeur. Il me paraît essentiel que la coopération scientifique internationale fournisse les connaissances et les arguments pour réduire l'usage de ces substances en production animale.

Travaillant dans des pays en développement, dotés de peu de moyens et d'infrastructures pour garantir la sécurité des aliments, j'envisage de poursuivre mes recherches sur la décontamination des produits carnés. Dans ce domaine, je suis partenaire avec le département de Biochimie de l'université d'Antananarivo d'un projet européen (**KBBE 2010-2013 intitulé : African food tradition revisited by research, 3 700 000 euros**) coordonné par mon UMR (Cirad) pour caractériser la qualité sanitaire du Kitoza, une viande séchée traditionnelle à base de porc ou de zébu. Nous envisageons de caractériser le mode de transformation artisanal et la qualité microbiologique des produits ainsi obtenus. Nous étudierons l'impact d'un **nouveau procédé consistant en un salage et un séchage simultanés par déshydratation imprégnation par immersion (DII) dans une solution de sel et de sirop de glucose suivis d'une fermentation lactique par *Lactobacillus sakei***. Les deux opérations seront réalisées à température ambiante (25˚C) afin de répondre aux conditions tropicales. L'objectif sera de pré-stabiliser le produit avant la fermentation, étape particulièrement propice au développement microbien incluant les contaminants pathogènes. Ce travail sera réalisé dans la continuité de la collaboration avec le Cirad à la Réunion (UMR Qualisud) pour évaluer l'intérêt de ce procédé sur la qualité sanitaire du Kitosa. Les travaux de cette équipe ont montré que lors de la DII, il y a un antagonisme entre le sucre (sirop de glucose) et le sel, ce qui limite la diffusion du sel ainsi que celle du sucre (Collignan *et al.*, 2001 ; Santchurn *et al.*, 2007). Cette équipe de l'UMR travaillera également à la compréhension et la modélisation des transferts de matière (eau, sel, sucres et acides) entre la surface et le cœur de l'aliment ainsi que leur couplage avec les cinétiques réactionnelles ayant lieu à la surface du produit (développement microbien, consommation de sucres, métabolisation d'acides).

Les différentes innovations évoquées précédemment ne pourraient être appliquées sans s'intéresser au contexte général du contrôle des denrées alimentaires dans les pays tropicaux. En effet, il serait vain d'innover sur les aspects technologiques de la production des aliments sans assurer les exigences basiques de la qualité sanitaire que sont l'application des bonnes pratiques d'hygiène. **L'analyse du système de contrôle des aliments à Madagascar a permis de mettre en évidence les lacunes communes aux pays en développement, à savoir le manque de volonté politique et de ressources pour mettre en place des normes alimentaires et les faire respecter**. Dans le contexte actuel, l'HACCP est loin d'être adopté pour le marché local. Aussi, nous avons préconisé l'élaboration de guides de bonnes pratiques d'hygiène pour aider les professionnels à la mise en œuvre des principes généraux de l'hygiène. Le manque d'expertise et d'éducation général rend cette tâche très difficile pour mettre en place des programmes efficaces. C'est pourquoi j'ai mené une réflexion sur l'élaboration et la mise en place de dispositifs adaptables aux conditions de terrain (cas des gargotes à Antananarivo).

J'ai donné **la priorité au montage de projets régionaux et internationaux** pour permettre au département de Biochimie de l'université d'Antananarivo qui m'a accueillie, **d'intégrer des réseaux scientifiques**. J'ai ainsi monté et géré le Pôle d'Excellence Régional financé par l'AUF avec les universités de la Réunion et des Comores ainsi que le Cirad (URP Forêt et Biodiversité à Madagascar et UMR Qualisud à Montpellier). Les activités de ce pôle pourront être poursuivies dans le cadre d'Horizons Francophones, un nouveau réseau universitaire régional financé par l'AUF qui portera sur la Biodiversité et les maladies émergentes (en cours de construction au niveau régional et incluant les partenaires de Madagascar et de la Réunion). J'ai aussi favorisé la participation de l'équipe malgache comme partenaire au projet Européen KBBE coordonné par mon UMR à Montpellier. Ce décloisonnement de la recherche me paraît essentiel pour renforcer les capacités des universités du sud. Pour ce faire, il a fallu équiper le laboratoire de microbiologie du département de Biochimie sur les différents projets qui ont ainsi financé près de 20000 euros d'achat (poste de sécurité microbiologique, 2 étuves microbiologiques, autoclave de paillasse, pHmètre, 2 balances, vortex, onduleur).

Le Cirad demande également à ses agents de générer des ressources propres. J'ai donc développé une expertise sur la sécurité des aliments en gardant le lien avec mes sujets de recherche. J'ai été consultante depuis 2008 (120 jours) pour le compte du Centre Technique

Horticole de Tamatave afin de **mettre en place les guides de bonnes pratiques d'hygiène dans les filières de production de litchis, vanille et girofle.** Pour ce faire, j'ai conduit des analyses de risques pour déterminer les dangers microbiens le long de ces filières du stade de récolte des produits jusqu'à l'exportation finale en Europe.

9 - Productions scientifiques

9.1. - Publications dans revues internationales à facteur d'impact

Sarter S., Randrianarivelo R., Ruez P., Raherimandimby M. and Danthu, P. Antimicrobial effects of essential oils of *Cinnamosma fragrans* on the bacterial communities of the water rearing of *Penaeus monodon* larvae. Vector borne and zoonotic diseases, July 2010, in press.

Randrianarivelo R., Danthu P., Benoit C., Ruez P, Raherimandimby M. and **Sarter S**. Novel alternative to antibiotics in shrimp hatchery: Effects of the essential oil of *Cinnamosma fragrans* on survival and bacterial concentration of *Penaeus monodon* larvae. Journal of Applied Microbiology, 2010, 109, 642-650.

Sarter S, Sarter G. and Gilabert P. A Swot analysis of HACCP implementation in Madagascar. Food Control, 2010, 21: 253-259.

Randrianarivelo R., **Sarter S.**, Odoux E., Brat P., Lebrun M., Menut C., Romestand B., Andrianoelisoa H.S., Raherimandimby M. and Danthu P. Composition and antimicrobial activity of essential oils of *Cinnamosma fragrans*. Food Chemistry, 2009, 114: 680-684.

Lecompte J.-Y., Collignan A., **Sarter S.**, Cardinale E. and Kondjoyan A. Decontamination of chicken skin surfaces inoculated with *Listeria innocua, Salmonella* Enteritidis and *Campylobacter jejuni* by contact with a concentrated lactic acid solution. British Poultry Science 2009, 50(3): 307-17

Sarter S., Metayer I. and Zakhia N. Effects of mycotoxins, Aflatoxin B1 and Deoxynivalenol (DON), on the bioluminescence of *Vibrio fischeri.* World Mycotoxin Journal 2008, 1(2): 189-193

Lecompte, J.-Y., Kondjoyan A., **Sarter S.,** Portanguen S. and Collignan A. Effects of steam and lactic acid treatments on inactivation of *Listeria innocua* surface-inoculated on chicken skins. International Journal of Food Microbiology, 2008, 127: 155-161.

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Sarter S. and Zakhia N. Chemiluminescent and bioluminescent assays as innovative prospects for mycotoxin determination in food and feed. Luminescence 2004, 19: 345-351

EL-AALAM S., Pauss A. and Lebeault JM. High efficiency styrene biodegradation in a biphasic organic/water continuous reactor. Applied Microbiology and Biotechnology 1993, 39: 696-699.

Articles soumis:

- Food Research International
  Thermal and chemical treatments to control *Salmonella* spp. on meat and poultry surfaces. A review
  Sarter S., Kondjoyan A., Lecompte J.Y. and Collignan A.

- Journal of Ethnopharmacology:
  In vitro evaluation of antibacterial, antioxidant and cytotoxic activities of *Dilobeia thouarsii* Roemer and Schulte leaf and bark extracts.
  Razafintsalama E.V., **Sarter S.**, Randrianarivo H. R., Rakoto D.A.D., Rajaonarison F. J, Petit T. and Jeannoda V.

- Social Science & Medicine:
  Rules, resources and germs: understanding hygiene to promote GHP in cheap restaurants in Madagascar
  Sarter G. and **Sarter S.**

- Flavour and Fragrance Journal
  Variability of the essential oil composition of *Cinnamosma fragrans* and *Cinnamosma madagascariensis*, endemic plants from Madagascar
  Razafimamonjison G., Danthu P., Lebrun M., **Sarter S.**, Ramanoelina P.

9.2. - Publications dans revue à comité de lecture (sans facteur d'impact)

Kondjoyan A., Portanguen S., Lecompte J.Y., **Sarter S.** and Collignan A. Le cas des carcasses de volailles : Intérêt d'utiliser les traitements par de la vapeur d'eau seule ou combinée à de l'acide lactique pour décontaminer la surface des viandes. Viandes et Produits Carnés 2007, 26 (4): 110-114.

Dabat, M.H., Andrianarisoa B., Aubry C., Ravoniarisoa E., Randrianasolo H., Rakoto N., **Sarter S.**, Trèche S. Production de cresson à haut risque dans les bas fonds d'Antananarivo ? Vertigo, 2010, 10 (2). Journal électronique.

9.3. - Chapitres d'ouvrages

Sarter S. Microbial safety of cured vanilla beans. In: Vanilla, Medicinal and aromatic plants-Industrial profiles, Vol 47, CRC Press, Taylor & Francis group, Odoux and Grisoni eds. 2011, chapter 14, 229-236, NY

Behra O., Danthu P., **Sarter S**., Radaniela R., Fourcade C., Randrianarivelo R., Ranaivosoa B., and Arnal-Schnebelen B. Saro (*Cinnamosma fragrans* Baillon) essential oil: Application in health and medicine. In African natural plant products: new discovery and challenges in chemistry and quality, pp 485-494. H. Rodolfo Juliani, James E. Simon and Chi-Tang Ho eds. 2009, ACS symposium series; 1021. Oxford University Press.

Sarter S. and Guichard B. Bacterial antibiotic resistance in aquaculture. In: Aquaculture Microbiology and Biotechnology, 2009, Volume 1, 133-157; Didier Montet and Ramesh C. Ray eds. Science Publishers Inc, New Hampshire, USA. ISBN 1578085748, 9781578085743.

Sarter S. et Muchnik J. Les savoirs traditionnels et les biotechnologies artisanales. Nourrir 9 milliards d'hommes, 2005. Collectif -ADPF. ISBN 2-914935-48-X

Siriex L. et **Sarter S.** Qu'est-ce qu'un aliment ?
Nourrir 9 milliards d'hommes, 2005. Collectif -ADPF. ISBN 2-914935-48-X

9.4. - Guides de Bonnes Pratiques d'Hygiène

Sarter S., Guide de bonnes pratiques d'hygiène dans les filières de production et de transformation de vanille à Madagascar. Consultance pour le Centre technique Horticole de Tamatave (Madagascar). UE/ COM STABEX, 8ème Fonds Européen de Développement. Novembre 2010.

Sarter S., Guide de bonnes pratiques d'hygiène dans les filières de production et de transformation de girofle à Madagascar. Consultance pour le Centre technique Horticole de Tamatave (Madagascar). UE/ COM STABEX, 8ème Fonds Européen de Développement, Octobre 2010.

Sarter S., Guide de bonnes pratiques d'hygiène dans la filière litchis à Madagascar, de la récolte à l'expédition. Consultance pour le Centre technique Horticole de Tamatave (Madagascar). Mai 2008.

9.5. - Communications orales

Sarter S and Danthu P. Antibiotic resistance of bacteria in aquaculture: Development of novel antimicrobials based on essential oils. Oral Communication at the International Conference on Food security during challenging times. University Putra Malaisia, 5-7th July 2010, Selangor Malaysia.

Arnaud, E, **Sarter S.**, Kondjoyan A., Collignan A. Combined effect of steam and lactic acid treatments for inactivating *Salmonella enterica* Serovar Enteritidis on chicken skin. World Congress of Food Science and Technology IUFoST 2010, Cape Town, South Africa.

Sarter S. 1- "Food control improvements: strengthening good hygiene practices". 2- "Antibiotic resistance: its significance and impacts on health management" in Workshop on Veterinary Public Health - Food Safety control in the SADC region (Food originating from Livestock). Oral communications, SADC, 26th- 28th February 2008. Antananarivo, Madagascar.

Sarter S. " Bacterial antibioresistance: its implication in Better Management Practices". In Workshop on Best Management Practices (BMPs) for small-scale shrimps farmers. AIT-NACA, 28-30 May, 2007.Bangkok. Thaïlande

Sarter S. "Tools for hazard management in shrimps production and processing" and "Methods for bacterial antibioresistance determination: application to aquaculture fish". Seminar for Food safety and International Trade. The French-Thaï approach and EU regulation. 8th-9th sept 2004, Bangkok. Thaïlande

9.6. - Communications par affiches

Arnaud, E, **Sarter S.**, Kondjoyan A., Collignan A. Combined effect of steam and lactic acid treatments for inactivating *Salmonella enterica* Serovar Enteritidis on chicken skin. World Congress of Food Science and Technology IUFoST 2010, Cape Town, South Africa.

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10 – Références

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Annexe 1 : Curriculum vitae

Samira SARTER
Née le 19 mai 1966 (Maroc)
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CHERCHEUSE EN MICROBIOLOGIE ALIMENTAIRE

FORMATION

1994 : **POST-DOCTORAT DE MICROBIOLOGIE**
Laboratoire de Chimie Microbienne (ULP Strasbourg/URA CNRS)
Mise au point de méthodes de purification et d'analyse du facteur de virulence d'une souche bactérienne pathogène (*Pseudomonas aeruginosa*).

1993 : **DOCTORAT DE MICROBIOLOGIE**
Université de Technologie de Compiègne (UTC)
Sélection de bactéries capables de dégrader des composés xénobiotiques. Application pilote pour l'épuration d'effluents contaminés (financement industriel Monsanto et contrat de confidentialité).
Mention : Très honorable et Félicitations du jury

1989 : **DEA EN TECHNOLOGIE ALIMENTAIRE**
Université de Technologie de Compiègne (UTC)
Caractérisation biochimique de bio-produits (pâtes alimentaires)

EXPERIENCE PROFESSIONNELLE

- **Chercheuse en Qualité et Sécurité Sanitaire des Aliments au Cirad/ UMR Qualisud (depuis janv. 2002, en poste à Madagascar depuis août 2006)**

  Projets de recherche en cours :
  - Etude de l'activité antimicrobienne des extraits issus de la biodiversité végétale malgache
  - Recherche d'alternatives aux antibiotiques en aquaculture (huiles essentielles)
  - Identification des risques sanitaires microbiologiques dans les filières de production et de transformation des denrées alimentaires (agriculture péri-urbaine, épices, restauration de rue, viandes)

Enseignement (France, Vietnam, Madagascar)
- Agents pathogènes des aliments
- Gestion et contrôle de la qualité sanitaire dans les systèmes de production alimentaire

- **Responsable Qualité (France 1999-2001)**

  - **Société Gel-Pêche productrice à Madagascar et importatrice de plus de 3000T/an de crevettes tropicales**

  - Evaluation et sélection des usines de production de crevettes (pêche et aquaculture): Madagascar, Nigéria, Brésil, Afrique du Sud, Iran, Guyane Française, Inde
  - HACCP, bonnes pratiques d'hygiène, analyses microbiologiques de routine.
  - Optimisation des process de traitement et conditionnement des produits (chaîne du froid, traitement chimique, hygiène)

- **Consultante Qualité/Gescal International (Inde 1997-1998)**
  - Installation en Inde (Bombay) d'un laboratoire d'analyses dans une usine de produits de la mer pour l'exportation vers Union Européenne.
  - Mise en place HACCP et les bonnes pratiques.

- **Chargée d'étude à ERMC Environmental Resources Management Consultants-(Jordanie 1995-1996)**
  - Réalisation d'études environnementales (études d'impact, station d'épuration, réutilisation des eaux usées en agriculture).
  - Organisation de 2 sessions de formation régionales (Syrie, Jordanie, Egypte, Palestine, Israël) sur la gestion et la qualité des ressources en eau, à destination des agents de développement (financement de la coopération française).

- **Collaboratrice Qualité / Laboratoire d'Ecotoxicologie de l'INERIS (1992-1993)**
  Elaboration des rapports d'essais des tests d'écotoxicologie conformément à différentes normes internationales (ISO, AFNOR, CEE) et selon les Bonnes Pratiques de Laboratoire.

- **Enseignante à l'UTC Compiègne (1991-93)**
  Travaux pratiques de microbiologie : identifications bactériennes, cultures et cinétiques de croissance aérobie, fermentation.

LANGUES

Français. Anglais. Arabe.

PRODUCTIONS SCIENTIFIQUES

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Annexe 2 : Copie des articles parus dans des revues à facteur d'impact

(par ordre chronologique décroissant)

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Type: research-article

VECTOR-BORNE AND ZOONOTIC DISEASES
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ORIGINAL RESEARCH

Antimicrobial Effects of Essential Oils of *Cinnamosma fragrans* on the Bacterial Communities in the Rearing Water of *Penaeus monodon* Larvae

Samira Sarter,[1] Roger Randrianarivelo,[2] Philippe Ruez,[3] Marson Raherimandimby,[4] and Pascal Danthu[5,6]

Abstract

Farmed shrimps are vectors of various *Vibrio* species that are considered a potential health hazard. Previous study has shown that *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio alginolyticus* dominated in the water and larval samples of shrimp hatchery (Randrianarivelo et al. 2010). The effects of two essential oils (EOs) of *Cinnamosma fragrans*, an endemic plant to Madagascar (B8: linalool-type and B143: 1,8-cineole-type), were determined on the total heterotrophic aerobic bacteria and the *Vibrio* concentrations in the rearing water of *Penaeus monodon* hatchery. The assays took place in OSO Farming's shrimp hatchery in Madagascar. EOs were directly added to the water tank. The bacterial concentrations of water tank were assessed on marine agar and thiosulfate citrate bile sucrose agar. The larvae culture corresponded to four replicates each of B8, B143, erythromycin (E), and control (oil and antibiotic free). The bacterial concentration of the rearing water in B8, B143, and antibiotic (E) tanks were significantly lower ($p < 0.05$) than in the control. Further, there was no significant difference ($p > 0.05$) between the three treatments B8, B143, and E. This study demonstrated that both EOs of *C. fragrans*, like antibiotic, inhibited bacterial growth in the rearing water of *P. monodon* larvae. The potential of *C. fragrans* EO to control the bacterial load in *in vivo* conditions of *P. monodon* hatchery makes it a relevant option for producers to minimize risk of *Vibrio* growth in the rearing water of larvae, which is the primary source of colonization of shrimp larvae.

AU2 ▶ **Key Words:** Madagascar.

Introduction

Pathogenic *Vibrio* spp. such as *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus* are human pathogens that are often isolated from fish or their immediate environment (Bhaskar et al. 1998, ICMSF 2005, Chinabut et al. 2006). Vibriosis is a major constraint on the intensive production of shrimps as Vibrionaceae family is one of the most important groups in marine environments and the major pathogenic bacteria for penaeid crustacean larvae (Bourne et al. 2004, Chrisolite et al. 2008). As these bacteria are common in the marine environment, the culture pond serves as a constant source of exposure for the shrimp. Massive mortalities of shrimp larvae associated with luminescent strains of *Vibrio* spp. have been reported in hatcheries from several countries (Lavilla-Pitogo et al. 1998, Sung et al. 2001). However, the intensive use of antibiotics has led to bacterial resistance, which became a high concern for both shrimp pond management and human health (Le and Munekage 2004, Sarter et al. 2007). In 1991, an epidemic of *V. cholerae* 01 infections affected Latin America. The epidemic strain in Latin America was susceptible to the 12 antimicrobial agents tested, but in coastal Ecuador, the epidemic strain had become multiresistant. Cholera epidemic in Ecuador began among persons working in shrimp farms. The multiresistant phenotype was present in noncholera *Vibrio* infections that were

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pathogenic to the shrimp. The authors suggested that resistance may have been transferred to *V. cholerae* 01 from other vibrios (Weber et al. 1994). In fact, potential transfer of resistant bacteria or resistant genes from animals to humans may occur through the food chain because reservoirs of antibiotic resistance can interact between different ecological systems (van den Bogaard and Stobberingh 2000, Witte 2000, Teuber 2001). Consequently, the contamination of the environment with bacterial pathogens resistant to antimicrobial agents is a potential threat not only as a source of disease but also as a source from which resistance genes can easily spread to other pathogens of diverse origins.

Therefore, alternative methods need to be developed to maintain a healthy microbial environment in the larval rearing tanks. It is recognized that rearing water is the main source for entry of pathogens in the host (Lavilla-Pitogo et al. 1998). In this regard, plant secondary metabolites, for example, essential oils (EOs), are known to display antimicrobial properties (Direkbusarakom et al. 1998, Dorman and Deans 2000, Elgayyar et al. 2001). The use of EOs of *Cinnamosma fragrans*, B143 and B8, which were characterized by their high content in oxygenated monoterpenes, 1,8-cineole and linalool, respectively, exhibited, like erythromycin, significant higher survival and lower bacterial concentrations of the larvae of *Penaeus monodon* than the control (Randrianarivelo et al. 2010). The present study aimed to show the effects of both EOs of *C. fragrans* on total heterotrophic bacterial and *Vibrio* spp. population concentrations in the rearing water of *P. monodon* larvae. Plant extracts have been generally used through oral administration using bioencapsulation, by direct mixture with feed ingredients, or by feeding to *Artemia*, which acts as a biological carrier that can then be fed to shrimps (Immanuel et al. 2004). From this point of view, use of EOs as antimicrobials in aquaculture, directly in the water tank, is innovative.

Materials and Methods

EO extraction

C. fragrans leaf samples were harvested in Mariarano for B143 sample (15°41′S: 46°43′E, altitude: 7 m, 71.6% 1,8-cineole) and in Tsaramandroso for B8 sample (16°18′S; 47°02′E, altitude: 600 m, 95.8% linalool) and were extracted as described by Randrianarivelo et al. (2009).

EO and antibiotic assays

The *in vivo* experiments were carried out at the OSO organic shrimp hatchery in Madagascar. The Nauplii larvae were cultured for 18 days in duplicate, and the corresponding larval stages were, respectively, Nauplii (N1, N6), Zoe (Z1, Z1Z2, Z2, Z3, Z3M1), Mysis (M1, M2, M3, M3PL1), and postlarvae (PL1, PL2, PL3, PL4, PL5, PL6, PL7, PL8) from T_0 till T_{18}. Sea water was filtered and sterilized by a ultraviolet system. Initially, a water volume of 9 L was maintained, with daily addition of 0.75 L during the first 7 days. To renew the water, the water exchange rate used was 25% by the 11th day (stage PL1), 50% by the 12th day (stage PL2), 50% by the14th day (stage PL4), and 50% by the 16th day (stage PL6). The aquaria were inoculated with 400 larvae per liter. The culture conditions maintained were 29.2°C, 32% salinity, pH 7.8, and 4.5%–5.5% of dissolved oxygen levels. Feeding was carried out according to the official "AB Label" organic standards applied by the farm protocol. The treatment (EO or antibiotic [erythromycin]) (Bouchara) was applied once a day at 7 a.m., on days 3, 5, 7, 10, 12, and 14. The antibiotic concentration was 20 μg mL^{-1}. The oil was added at a concentration of 734 μg mL^{-1}. It was directly mixed with water. For the assays using Tween 80 (Fisher Labosi), the oil was mixed in a solution of 0.04% (v/v) Tween 80. In the control B, a solution of only emulsifier (Tween 80) was added at 0.04% (v/v) at the same frequency as the oil assays.

The following assays were performed in duplicate and during two campaigns in 2006 and 2007 as described by Randrianarivelo et al. (2010):

1. Control A: the larvae culture was not treated (no EOs, no antibiotic, no Tween 80).
2. Control B: the larvae culture was treated with Tween 80 (no EOs, no antibiotic).
3. B8 T: the larvae culture was treated with B8 EO with Tween 80.
4. B8: the larvae culture was treated with B8 EO without Tween 80.
5. B143 T: the larvae culture was treated with B143 EO with Tween 80.
6. B143: the larvae culture was treated with B143 EO without Tween 80.
7. E: the larvae culture was treated with the antibiotic erythromycin.

Bacterial concentration

The culture started from Nauplii stage (day 0) till PL8 (day 18). Every day during the 18 days of *P. monodon* culture, water (10 mL) from each aquarium was sampled. The water samples were diluted to 10^{-5} in sterile saline solution. A volume of 0.1 mL from each dilution was plated in duplicate onto marine agar (MA) for total heterotrophic aerobic bacteria (Difco) and onto thiosulfate citrate bile sucrose (TCBS) agar for vibrios (Difco) and incubated at 30°C for 48 h and 24 h, respectively.

Statistical analysis

Kruskal–Wallis test was used to determine the significant difference between the tested groups at a threshold of $p < 0.05$. The reproducibility of the data collected in 2006 and 2007 was validated by Kruskal–Wallis test with a threshold of $p < 0.05$. As the analysis of data collected in duplicate for 2006 and 2007 has demonstrated that all p-values were >0.05, the data presented in this article corresponded to both years 2006 and 2007 (four replicates).

Results

The bacterial concentration of the rearing water in both the EOs and antibiotic (E) tanks were significantly lower ($p < 0.05$) than in the control (Figs. 1 and 2). Further, there was no significant difference ($p > 0.05$) between the three treatments (B8, B143, and E). At day 18, the $\log_{10}$ reduction between the control and the three treatments were 0.32 $\log_{10}$ cfu mL^{-1} on MA and 0.93 $\log_{10}$ cfu mL^{-1} on TCBS. ◀F1◀F2

In all assays, use of an emulsifier (Tween 80) did not have a significant effect on the bacterial concentrations of the water ($p > 0.05$) (Fig. 3). ◀F3

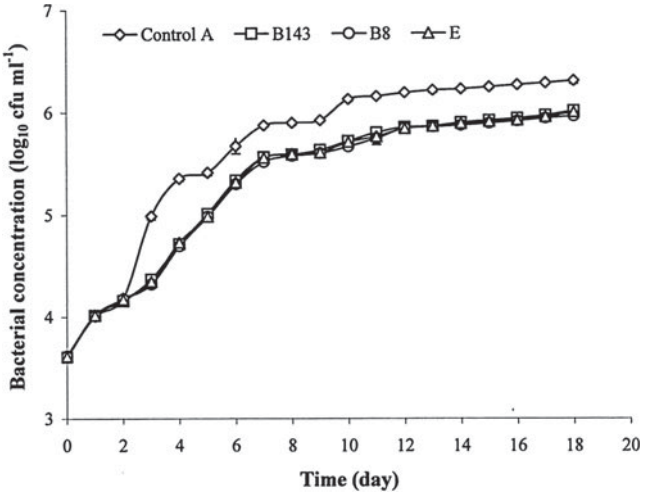

FIG. 1. Evolution of total bacterial concentration of rearing water on MA ($\log_{10}$ cfu mL^{-1}) during 18 days of culture of *Penaeus monodon* larvae for the control A (essential oil and antibiotic free) and for assays using the essential oils of *Cinnamosma fragrans* (B8 and B143) and erythromycin (E). Values correspond to the mean of four replicates ($n = 4 \pm$ SD). MA, marine agar; SD, standard deviation.

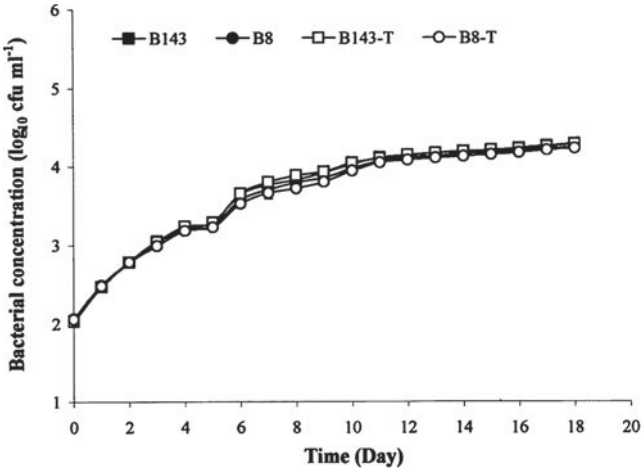

FIG. 3. Evolution of *Vibrio* concentration of the rearing water of *P. monodon* larvae on TCBS ($\log_{10}$ cfu mL^{-1}), for assays using essential oils of *C. fragrans* with Tween 80 (B8-T and B143-T; white symbols) and without Tween 80 (B8 and B143; black symbols). No significant difference was found between B8/B8-T and B143/B143-T ($p > 0.05$). Values correspond to the mean of four replicates ($n = 4 \pm$ SD). ◀AU6

Discussion

Total bacterial counts were consistently higher on the general heterotrophic medium (MA) than on the *Vibrio*-selective TCBS, indicating the presence of non–*Vibrio*-related microorganisms in the aquaculture system. During larval rearing, different microflora may have come into hatchery systems through live feeds such as algae and *Artemia* (Hameed and Balasubramanian 2000, Otta et al. 2001, Kennedy et al. 2006). Increasing *Vibrio* population in larvae and rearing tank water has been reported to reduce the survival rate of larvae and postlarvae (Regunathan and Wesley 2004). Previous results have shown that isolates on TCBS from water and larval samples included a variety of vibrios species, among which *V. alginolyticus*, *V. parahaemolyticus*, and *V. vulnificus* were dominant (Randrianarivelo et al. 2010). These species have been reported as pathogenic agents potentially transmitted to man from fish and fish products (ICMSF 2005).

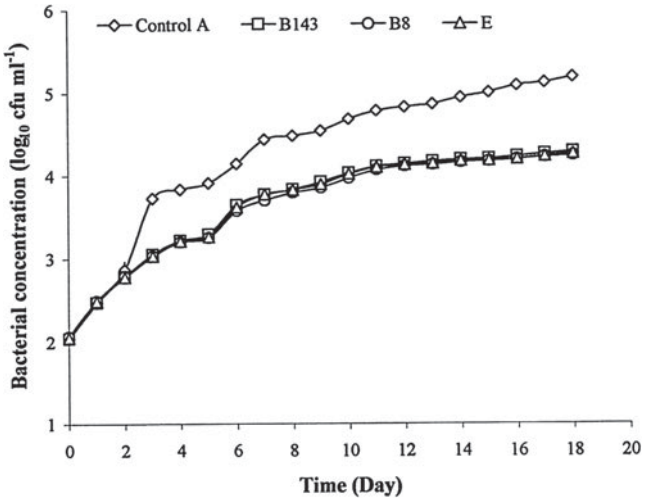

FIG. 2. Evolution of *Vibrio* concentration of rearing water on TCBS ($\log_{10}$ cfu mL^{-1}) during 18 days of culture of *P. monodon* larvae for the control A (essential oil and antibiotic free) and for assays using the essential oils of *C. fragrans* (B8 and B143) and erythromycin (E). Values correspond to the mean of four replicates ($n = 4 \pm$ SD). TCBS, thiosulfate citrate bile sucrose.

In all assays, use of an emulsifier (Tween 80) did not have a significant effect on the bacterial concentration of the water ($p > 0.05$), which is in accordance with previous results obtained with *P. monodon* larvae (Randrianarivelo et al. 2010). Both EOs inhibited the bacterial growth in *in vivo* conditions of larval culture, like the antibiotic (Figs. 1 and 2). The $\log_{10}$ reduction was higher for *Vibrio* population than for total bacteria for the three treatments (EOs and antibiotic). This may be attributed to the antimicrobial effects of EOs as demonstrated by their low minimum inhibitory concentration against different *Vibrio* species (Randrianarivelo et al. 2009, 2010). Terpenes have been reported to cause disruption of bacterial membranes by leakage of intracellular constituents (Ultee et al. 1999, Di Pasqua et al. 2007). Because of their mode of action affecting several targets, no particular resistance or adaptation to EOs has been described so far in the literature (Bakkali et al. 2008).

Several studies have reported the potential to use plant extracts as an alternative to antibiotics in aquaculture and in organic farming in particular. Juveniles of *Penaeus indicus* fed with enriched diets (from terrestrial plants: *Ricinus communis*, *Phyllanthus niruri*, *Leucus aspera*, *Manihot esculenta*; and sea weeds: *Ulva lactuca* and *Sargassum wightii*) had better survival and growth, with inhibition of bacterial load of *V. parahaemolyticus* (Immanuel et al. 2004). The green algae *Tetraselmis suecica*, which has been reported to inhibit *in vitro* growth of pathogenic *Vibrio* spp., reduced *Vibrio* count in both rearing water and larval samples when it was used as feed in

larval rearing tanks of the prawn *Fenneropenaeus indicus* (Indian white shrimp, *Penaeidae*) (Regunathan and Wesley 2004). *Stevia* plant extracts increased the immunity and disease resistance of *P. monodon* shrimp (Leaño et al. 2007). Similarly, the hot-water extracts from leaves and twigs of *Camphor kanekirae* have also shown better immunity of *Litopenaeus vannamei* shrimp and disease resistance to *V. alginolyticus* (Yeh et al. 2008). The protection of shrimps elicited by *Dendrilla nigra*, a marine sponge, against the most common shrimp pathogens such as luminescent *Vibrio harveyi* and *V. alginolyticus* could be due to its antibiotic effect rather than its influence on the host defense system (Selvin and Lipton 2004).

Use of EOs of *C. fragrans* in water tank have been found to be effective in reducing significantly the bacterial load of both heterotrophic bacteria and *Vibrio* spp. in *in vivo* conditions of larval culture. This might be a relevant option for producers to control the microbial growth in the rearing water, and *Vibrio* population in particular, using natural plant extracts rather than antibiotics. *C. fragrans* is a common species in the western forests of Madagascar. It is not indicated as a threatened AU3 ▶ species on the IUCN Red List (IUCN 2009). Moreover, EOs are extracted from leaves, which is not a destructive method. These reasons allow us to envisage a sustainable exploitation of this resource in Madagascar.

AU4 ▶ **Disclosure Statement**

No competing financial interests exist.

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ORIGINAL ARTICLE

Novel alternative to antibiotics in shrimp hatchery: effects of the essential oil of *Cinnamosma fragrans* on survival and bacterial concentration of *Penaeus monodon* larvae

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Keywords
antimicrobial activity, aquaculture, *Cinnamosma fragrans*, essential oil, *Penaeus monodon*.

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Abstract

Aims: The activity of two essential oils (EOs) of *Cinnamosma fragrans*, an endemic plant to Madagascar (B8: linalool-type and B143: 1,8-cineole-type), against bacterial isolates from a shrimp hatchery of *Penaeus monodon* and their effects on the survival and bacterial concentration of larvae were determined.
Methods and Results: Minimum inhibitory concentrations were determined using a broth dilution technique. The bacterial concentrations of both larvae and water tank were assessed on Marine agar and Thiosulfate Citrate Bile Sucrose agar. The assays took place in OSO Farming's shrimp hatchery in Madagascar. EOs were directly added to the water tank. Regarding the survival, the assays in larval culture (four replicates each of B8, B143, E and control) showed that B8 oil had a similar effect ($P > 0{\cdot}05$) as the antibiotic (Erythromycin) and was more active than B143 ($P < 0{\cdot}05$). A negative correlation was observed between the bacterial concentration and the survival of larvae for all assays.
Conclusion: Both *C. fragrans* essential oils, as antibiotic, exhibited significantly higher survival rates and lower bacterial concentrations of the larvae than the control (oil and antibiotic free).
Significance and impacts of the study: The potential of *C. fragrans* essential oil to control the bacterial load in *in vivo* conditions, thereby enhancing survival rate of *P. monodon* larvae, makes it a relevant option for developing a novel alternative to antibiotics in shrimp hatchery culture.

Introduction

The worldwide application of antibiotics in aquaculture for prophylactic and therapeutic purposes has resulted in an increase in bacterial resistance in exposed microbial ecosystems, impacting both animal and public health. International agencies recommend that antibiotics should be restricted to therapeutic purposes only, and that preventative approaches to disease management should be preferred over costly posteffect treatments (GESAMP 1997; FAO 2005). Therefore, to make the aquaculture industry more sustainable, new strategies to control infection are urgently needed (Defoirdt *et al.* 2007; Hsieh *et al.* 2008; Parisien *et al.* 2008).

Among alternatives to antibiotics in shrimp aquaculture, use of plant extracts as immunostimulants for enhancement of nonspecific, host defence mechanisms or as antimicrobials for bacterial growth inhibition has been reported as a relevant strategy. (Direkbusarakom *et al.* 1998; Dorman and Deans 2000; Citarasu *et al.* 2002; Leaño *et al.* 2007; Yeh *et al.* 2008). Use of plant extracts to replace chemotherapeutics in aquaculture has been generally achieved through oral administration (bioencapsulation) or direct mixture with feed ingredients. As the plant extracts are nonpalatable

to shrimps, they could be first fed to *Artemia*, which acts as a biological carrier (Immanuel *et al.* 2004).

Cinnamosma fragrans, an endemic species of Madagascar, belonging to the Canellaceae family grows in the tropophytic forests though is often found near the coast as well. Randrianarivelo *et al.* (2009) demonstrated that 1,8-cineole and linalool were the main constituents of essential oil samples of *C. fragrans* originating from these two geographical regions of Madagascar. In describing the chemical composition of this species' extracts, Schulte *et al.* (1972) assumed that linalool is the main compound in the essential oil isolated from *C. fragrans* leaves. Recently, 26 constituents were identified for *C. fragrans* of which 1,8-cineole (51·0%) and sabinene (10·6%) were the major components (Tucker *et al.* 2008).

Cinnamosma fragrans is used traditionally against respiratory, parasitic and gastrointestinal infections, syphilis and malaria (Pernet and Meyer 1957; Schulte *et al.* 1972; Milijaona *et al.* 2003). Its essential oil from leaves exhibited strong antimicrobial activity against Gram-negative bacteria, including several vibrio strains specific to aquaculture (Randrianarivelo *et al.* 2009). These results could presume consideration of this EO for application in shrimp aquaculture to confirm *in vitro* activities *in vivo,* determining whether there is a difference because of chemical type. This knowledge is important for planning sustainable management of such an endemic species, as well as to initiate selection of the most interesting chemotype for large scale utilisation as with other species of Madagascar (Danthu *et al.* 2010). *Cinnamosma fragrans* is a common species in the western forests of Madagascar. It is not indicated as a threatened species on the IUCN Red List (IUCN, 2009). Moreover, essential oils (EOs) are extracted from leaves, which is not a destructive method. These reasons allow us to envisage a sustainable exploitation of this resource in Madagascar.

The objective of this work was to determine the effects of two chemical types (B8 and B143) of the EOs of *C. fragrans* on the survival and the bacterial concentration of larvae (total bacteria and vibrios) of *Penaeus monodon* in comparison with the control and the antibiotic: Erythromycin. The minimum inhibitory concentrations (MICs) of both oil samples were determined for bacterial isolates from the shrimp hatchery. The effect of an emulsifier was also evaluated to optimize feasibility of use of EOs in an aquatic medium.

Material and methods

Essential oil extraction

Cinnamosma fragrans leaf samples were collected during the rainy season, in February 2005: essential oil sample B143 was harvested in Mariarano (15°41′S: 46°43′E, altitude: 7 m) and B8 sample in Tsaramandroso (16°18′S; 47°02′E, altitude: 600 m). The leaves were steam distilled for 4 h in a Clevenger-type apparatus, <24 h after sampling. The EOs were dried over anhydrous sodium sulfate until any trace of water was removed, and then stored in opaque glass bottles at 4°C as described by Randrianarivelo *et al.* (2009).

Determination of minimum inhibitory concentration (MIC)

The activity of the essential oil samples was tested for different bacterial isolates from the *P. monodon* hatchery of OSO Farming (Antsiranana Province, Madagascar) and two reference strains, *Vibrio penaeicidae* (ATCC 51841) and *Vibrio splendidus* (ATCC 25914) that were given by Ifremer (Montpellier, France).

MIC (minimum inhibitory concentration as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth) and MBC (minimum bactericidal concentration as the lowest concentration at which no growth occurred on the agar plates) were determined in triplicate, using a broth dilution method for samples B8, B143, pure linalool and 1,8-cineole as described by Randrianarivelo *et al.* (2009). Pure linalool and 1·8-cineole were purchased from Aldrich Chimie (Saint Quentin Fallavier, France).

Stock solutions of EOs were prepared in sterile distilled water. These suspensions were further diluted from 0·04 mg ml^{-1} to 23·5 mg ml^{-1} in test tubes. And 30°C-overnight cultures were inoculated in 900 μl of Zobell (Maes and Paillard 1992) for *Vibrio* strains or Poor broth (Destoumieux *et al.* 1999) for the other strains. The optical density of the inoculum was measured at D_{600} = 0·1 and then diluted to reach a final optical density of D_{600} = 0·001 in the assay. To these cultures, 100 μl of the essential oil dilution were then added to reach a final volume of 1 ml. A positive control containing the bacterial culture without the essential oil and a negative control containing only the medium were also established. Tubes were incubated 24 h at 25°C for *Vibrio* spp., 30°C for *Micrococcus* spp., and 37°C for *Bacillus* spp. and their optical density was measured after incubation at D_{600}.

Essential oil and antibiotic assays

The *in vivo* experiments were all carried out at the OSO organic shrimp hatchery in Madagascar. The Nauplii larvae were cultured during 18 days in duplicate. The assays were carried out twice in 2006 and 2007. Sea water was filtered and sterilised by a UV system. Initially, a water volume of 9 l was maintained, with daily addition

of 0·75 l during the 7 first days. The water lost from the aquarium through evaporation was replenished by sterile sea water. The water exchange rate was then 25% by the 11th day (stage PL1), 50% by the 12th day (stage PL2), 50% by the14th (stage PL4) and 50% by the 16th day (stage PL6). Aquarium was individually provided by continuous aeration and light in neon. The aquaria were inoculated with 400 larvae per litre. The culture conditions were maintained at 29·2°C; salinity of 32‰; pH 7·8; 4·5–5·5% of dissolved oxygen levels. Feeding was carried out according to the official 'AB Label' organic standards applied by the farm protocols (Anon 2007). Larvae were reared on a mixed diet of resuspended *Chaetoceros gracilis* paste, Nauplii of *Artemia* and Frippak micro-capsules.

Effects of the EOs (B8 and B143 samples) and the antibiotic [Erythromycin (E), Bouchara Levallois, France] were investigated on the bacterial concentration of both larvae and water tank and on the survival rate of larvae during the 18 days of the development cycle. Tween 80 was tested as an emulsifier for oils to compare its effects to Tween-free oil assays.

The following assays were performed in duplicate and during two campaigns in 2006 and 2007:

1. Control A: the larvae culture was neither treated (no EOs, no antibiotic, no Tween 80).
2. Control B: the larvae culture was conducted with Tween 80 (no EOs, no antibiotic).
3. B8 T: the larvae culture treated with B8 essential oil with Tween 80.
4. B8: the larvae culture treated with B8 essential oil without Tween 80.
5. B143 T: the larvae culture treated with B143 essential oil with Tween 80.
6. B143: the larvae culture treated with B143 essential oil without Tween 80.
7. E: the larvae culture treated with antibiotic Erythromycin.

The treatment (essential oil and antibiotic) was applied once a day at 7 AM, on the following days: 3, 5, 7, 10, 12 and 14. The antibiotic concentration was 20 μg ml^{-1}. The oil was added at the concentration of 734 μg ml^{-1} (corresponding to the lowest MIC of oil against tested vibrios as determined by Randrianarivelo *et al.* (2009). For the assays using Tween 80, the oil was mixed in a solution of 0·04% (v/v) of Tween 80.

In the control B, a solution of only the emulsifier (Tween 80) was added at 0·04% (v/v) at the same frequency as the oil assays.

Bacterial concentration and strain isolation

Every day during the 18 days of the *P. monodon* culture, both larvae (15 larvae) and water (10 ml) from each aquarium were sampled. *Penaeus monodon* larvae were crushed into 5 ml of sterile saline water (2·5% NaCl in distilled water). The homogenized sample was serially diluted to 10^{-5} in sterile saline solution. The water samples were diluted in sterile saline solution as well. A volume of 0·1 ml from each dilution from both larvae and water samples were plated onto Marine agar (MA) for total heterotrophic aerobic bacteria (Difco, Le Pont de Claix, France) and onto TCBS agar for vibrios (Thiosulfate Citrate Bile Sucrose agar; Difco), both in duplicate, and incubated at 30°C during 48 h for MA and 24 h for TCBS. About three to five colonies growing on TCBS agar media were randomly isolated from samples (water and larvae) every day during the 18 days. A total of 80 strains were finally purified by successive streaking on TCBS media. The preliminary identification was carried out by Gram staining, oxidase and catalase reactions, microscopic observation, cell motility, glucose metabolism. The Gram-negative isolates were then identified by API System strips (Biomérieux at Marcy l'Etoile. France): API 20NE for bacilli Gram-negative oxidase positive (non-*Enterobacteriaceae*). All API strips were prepared according to the instructions of the manufacturer. The Gram-positive isolates were identified by Institut Pasteur of Lille (Lille, France) using API 50CHB for *Bacillus* spp. and APISTAPH for *Micrococcus* spp. The isolates were maintained on MA (Difco Laboratories, Detroit, MI, USA).

Larval survival rate

The larval survival rate was verified every day at 6 AM for all the experiments, with four 250 ml cultures counted visually. These larvae were restored to their original container to reduce mortality because of sampling. Only those larvae showing movement were considered alive.

The survival rate (%) was calculated as described by Baruah *et al.* (2009):

$$\text{Larval survival}(\%) = (\text{Total numbers of live larvae each day}/\text{Initial number of larvae}) \times 100$$

The toxicity of the EOs of *C. fragrans* was tested by bathing healthy larvae of *P. monodon* in treated seawater for 24 h. Batches of each of Nauplii, Zoe, Mysis, Postlarvae at a density of 100 per 1000 ml were reared in flasks containing sterile seawater (2000 ml) with varying concentrations of oil (0, 100, 200, 400, 700, 750, 800, 850, 900, 950, 1000 mg l^{-1}). The physicochemical characteristics of the rearing environment were salinity: 32‰, pH: 7·8, temperature: 29·2°C and dissolved oxygen: 4·5 mg l^{-1}. After 1 h of acclimation, the essential oil was added. Three replicates for each concentration of oil

were carried out. Live larvae were counted after 24 h of treatment, and survival rate was determined using the following formula (Yilmaz *et al.*, 2004):

$$\mathrm{LC50} = \mathrm{LC100} - \sum(\mathrm{ab})/n$$

LC50 and LC100 correspond to the lethal doses for the 50 and 100% of the samples, 'a' gives the difference between the two consecutive doses, 'b' the arithmetic mean of the mortality caused by two consecutive doses and 'n' the number of samples in each group.

Statistical analysis

Kruskal–Wallis test was used to determine the significant difference between the tested groups at a threshold of $P < 0{\cdot}05$. Before analysis, percent data were normalized using an arcsin transformation (Yeh *et al.* 2008). The reproducibility of the data collected in 2006 and 2007 was validated by Kruskal–Wallis test with the threshold of $P < 0{\cdot}05$. As the analysis of the data collected (bacterial concentration and survival percentage) in duplicate for 2006 and 2007 have demonstrated that all *P*-values were higher than 0·05, the data presented in this article corresponded to both years 2006 and 2007 (four replicates).

The correlation at each stage between the bacterial concentration and the survival of larvae was analysed using Spearman's correlation coefficient method.

Results

The culture spanned from Nauplii stage (day 0) to post-larvae PL 8 (day 18). The initial mean bacterial concentrations at T0 ($n = 16 \pm$ SD) were $8{\cdot}67 \times 10^3 \pm 1{\cdot}58 \times 10^2$ CFU ml^{-1} on MA (Fig. 1) and $1{\cdot}53 \times 10^2 \pm 3{\cdot}69$ CFU ml^{-1} on TCBS (Fig. 2). The isolated microflora from larvae ($n = 48$) consisted mainly of species of the genus *Vibrio*. From the water samples ($n = 32$), the isolates were mainly represented by *Vibrio* spp. and *Micrococcus* spp. (Table 1).

The MBCs values of B8 (95·8% linalool) were equivalent to the MICs values (bactericidal effect) for *Bacillus pumulus*, *B. subtilis, Micrococcus* spp., *Vibrio penaeicidae, V. parahaemolyticus, V. vulnificus* and *Photobacterium damselae* B 143 sample (71·6% 1,8-cineole) was bactericidal for *V. penaeicidae, V. splendidus, V. hollisae, V. alginolyticus V. parahaemolyticus, V. vulnificus and Photobacterium damselae* (Table 2).

Among Gram-positive strains, *Micrococcus* spp. was the most resistant strain for both B8 and B143. Among Gram-negative, the most resistant strains were *Vibrio alginolyticus* and *V. vulnificus* to B8 and *V. parahaemolyticus*

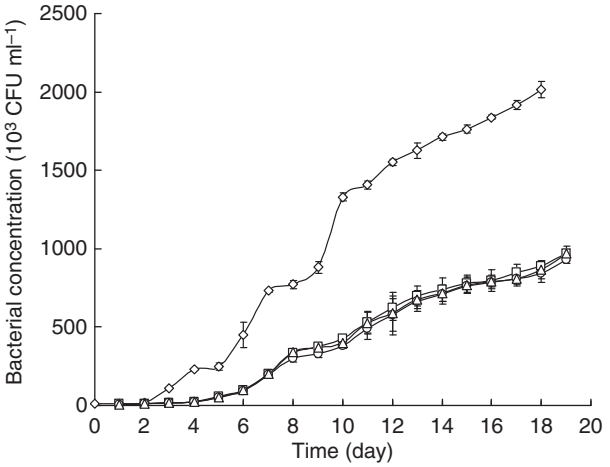

Figure 1 Evolution of total bacterial concentration (CFU ml^{-1}) of *Penaeus monodon* larvae during 18 days of culture for the control A (essential oil and antibiotic free) and for assays using the essential oils of *Cinnamosma fragrans* (B8 and B143) and Erythromycin (E). Values correspond to the mean of four replicates ($n = 4 \pm$ SD). (–◇–) Control A; (–□–) B143; (–○–) B8 and (–△–) E.

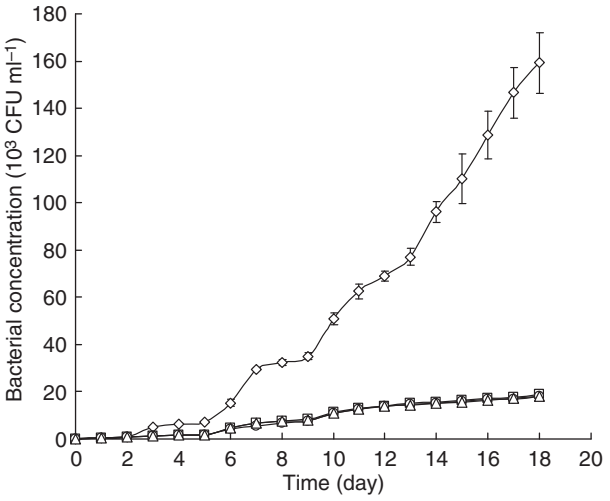

Figure 2 Evolution of *Vibrio* concentration (CFU ml^{-1}) of *Penaeus monodon* larvae during 18 days of culture for the control A (essential oil and antibiotic free) and for assays using the essential oils of *Cinnamosma fragrans* (B8 and B143) and Erythromycin (E). Values correspond to the mean of four replicates ($n = 4 \pm$ SD). (–◇–) Control A; (–□–) B143; (–○–) B8 and (–△–) E.

and *V. splendidus* to B143. B8 was more active than B143 towards *V. parahaemolyticus* and less active towards *V. alginolyticus.* B143 was the most active against *V. penaeicidae.* Towards the isolated strains, B8 and B143 oils exhibited the lowest MICs against *Bacillus pumulus* and *B. subtilis.* Globally, *C. fragrans* essential oil samples exhibited different MICs against the majority of strains than their respective pure major component, linalool and

Table 1 Identification of randomly selected bacterial isolates on TCBS medium from water (n = 32) and larvae (n = 48) samples of EOs assays and controls

Bacterial species	Water		Larvae	
	Nb isolates (n = 32)	(%)	Nb isolates (n = 48)	(%)
Bacillus pumulus	3	9	1	2
Bacillus cereus	2	6	1	2
Bacillus subtilis	2	6		
Micrococcus spp.	4	13		
Total Gram positive		34		4
Vibrio hollisae	2	6	7	15
Vibrio alginolyticus	7	22	13	27
Vibrio parahaemolyticus	2	6	5	10
Vibrio vulnificus	3	9	6	13
Photobacterium damselae	2	6	4	8
Vibrio spp.	5	16	11	23
Total Gram negative		66		96

1,8 cineole. B8 and B143 showed higher MICs than linalool and 1,8-cineole against *Vibrio alginolyticus* and *V. parahaemolyticus*, respectively.

Table 2 Minimum inhibitory concentration (mg ml^{-1}) of B8 and B143 samples of *Cinnamosma fragrans* essential oils from Tsaramandroso and Mariarano origins, respectively, and pure linalool and 1,8-cineole against shrimp farm isolates (n = 10) and two reference strains (ATCC)

Bacterial species	MIC (mg/ml)			
	B8 (95·8% linalool)	B143 (71·6% 1,8-cineole)	Linalool	1,8-cineole
Gram positive				
Bacillus pumulus	0·18a	0·37b*	0·18a	0·37b*
Bacillus cereus	0·73a*	1·47b*	1·47b	1·47b*
Bacillus subtilis	0·18a	0·37b*	0·18a	0·37b*
Micrococcus spp.	5·88a	5·88a*	5·88a*	11·75b
Gram negative				
Vibrio penaeicidae ATCC 51841	2·93b	0·18a	0·18a	2·93b
Vibrio splendidus ATCC 25914	2·93a*	5·88b	5·88b	2·93a*
Vibrio hollisae	0·73a*	0·73a	0·73a*	2·93b
Vibrio alginolyticus	5·88c*	2·93b	0·73a*	2·93b
Vibrio parahaemolyticus	2·93a	5·88b	5·88b	2·93a*
Vibrio vulnificus	5·88b	2·93a	5·88b	5·88b
Photobacterium damselae	2·93b	1·47a	1·47a	2·93b
Vibrio spp.	2·93a*	1·47a*	5·88b	1·47a*

MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration.

Values followed by different letters within a line are significantly different by Fisher LSD (P < 0·05).

*indicates that MBC > MIC.

Values without * indicate that MBC=MIC.

For each treatment (B8, B143, E) and control A, a significant correlation was observed between bacterial concentrations of both water and larval samples, with large correlation coefficients (r > 0·98) and associated P-values lower than 0·05 for each experiment (P < 0·0001). This article shows only the results related to the bacterial concentration of larvae (Figs 1 and 2). The bacterial concentration of larvae in B8, B143 and antibiotic (E) tanks were significantly lower (P < 0·05) than in the control (Figs 1 and 2). Furthermore, there was no significant difference (P > 0·05) between the three treatments (B8, B143 and E). Inversely, the survival in those assays was significantly higher than in the control (Fig. 3). The linalool-type oil (B8), which had similar effects than the antibiotic (E), was more active than the 1,8-cineole-type oil (B143). Among the three treatments (B8, B143, E), the survival of *P. monodon* larvae at PL 8 reached 82·6% with B8 and 80·5% with the antibiotic (E), followed by B143 at 69·3%, while the control had a survival rate of 15·1%.

A significant correlation was observed between the bacterial concentration of the larvae (for both total bacteria and vibrios counts) and the survival rate at all larval stages (r = −0·98 for the control; −0·97 for B143; and −0·96 for B8 and −0·96 for E; P < 0·05).

In all assays, use of an emulsifier (Tween 80) did not have a significant effect on the bacterial concentration of the larvae and their survival rate (P > 0·05) (Fig. 4).

The toxicity tests of B8 and B143 oils on Nauplii, Zoé, Mysis and Postlarvae showed that the mean value of

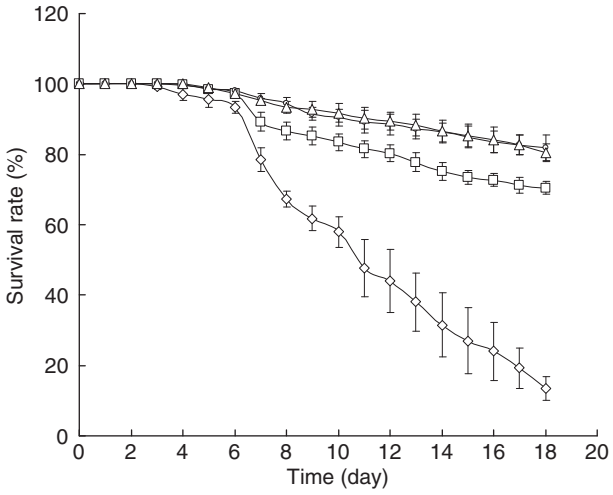

Figure 3 Evolution of the survival rate (%) of *Penaeus monodon* larvae during 18 days of culture for the control A (essential oil and antibiotic free) and for assays using the essential oils of *Cinnamosma fragrans* (B8 and B143) and Erythromycin (E). Values correspond to the mean of four replicates (n = 4 ± SD). (–◇–) Control A; (–□–) B143; (–○–) B8 and (–△–) E.

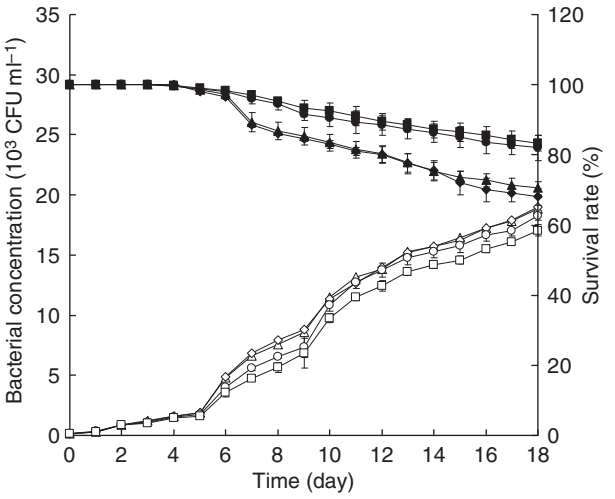

Figure 4 Evolution of the *Vibrio* concentration (CFU ml^{-1}) and the survival rate (%) of *Penaeus monodon* larvae, for assays using essential oils of *Cinnamosma fragrans* with Tween 80 (B8T, B143T) and without (B8, B143). White symbols correspond to bacterial concentration (CFU ml^{-1}) and black symbols to survival rate (%). No significant difference between B8/B8T and B143/B143T ($P > 0{\cdot}05$). (–△–) B143; (–◇–) B143T; (–□–) B8 and (–○–) B8T.

LC50 was significantly lower for B8 (863 mg l^{-1}) than B143 (885 mg l^{-1}) ($P < 0{\cdot}05$, Fisher LSD).

Discussion

Vibrionaceae family is part of the autochthonous flora of marine organisms and one of the most important groups in marine environments (Table 1). It is recognized that some *Vibrio* specie are difficult to identify at the species level using biochemical characters (Gopal *et al.* 2005). The specie found in this work participate to the diversity of the *Vibrio* community in *P. monodon* culture ponds (Leaño *et al.* 1998; Sung *et al.* 1999). Bacillaceae and Micrococcaceae isolates, which have been isolated on TCBS medium as well (López-Torres and Lizárraga-Partida 2001), have been reported in aquaculture systems (Nedoluha and Westhoff 1997), in *Artemia* and *Artemia* rearing water (López-Torres and Lizárraga-Partida 2001; Kennedy *et al.* 2006). Gram-negative bacteria are the major group isolated from healthy penaeid larvae and juveniles (Vandenberghe *et al.* 1998; Oxley *et al.* 2002). Both dominance of the *Vibrio* population in larvae and their higher resistance to tested oils may give the resident species a competitive edge over Gram-positive reproducing in the larvae. While in the water tank, feeding with *Artemia* may explain the higher presence of Gram-positive bacteria. Plants are generally considered as rich sources of safe and economical active compounds (Citarasu *et al.* 2002). The EOs extracted from the leaves of *C. fragrans* collected in two regions in Madagascar (B143 in Mariarano and B8 in Tsaramandroso) were characterized by their high content in oxygenated monoterpenes, 1,8-cineole and linalool, respectively (Randrianarivelo *et al.* 2009), which are known for their antimicrobial properties (Sivropoulou *et al.* 1997). Others minor constituents found have also been reported for their antimicrobial activity, such as p-cymene, α-pinene, *β*-pinene, limonene, α-terpinene, α–terpinolene, caryophyllene oxide and camphene (Sökmen *et al.* 2004). For the majority of the isolates, Bacillaceae were more sensitive than the Vibrionaceae isolates, which is in accordance with several reports (Dubber and Harder 2008). Gram-negative organisms possess an outer membrane, which may account for the increase in resistance to hydrophobic components (Griffin *et al.* 2001). Although the antimicrobial activities of EOs are well established, their mode of action is still poorly understood (Baser *et al.* 2006). Cytotoxic effects were observed *in vitro* in Gram-positive and Gram-negative bacteria (Burt 2004). Terpenes have been reported to cause the disruption of the membranes of *Bacillus thuringiensis* and *Bacillus cereus* causing the leakage of intracellular constituents (Andrews *et al.* 1980; Ultee *et al.* 1999). Oregano essential oil and two of its main components (thymol and carvacrol) were found to damage the membrane integrity of *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella typhimurium* (Knobloch *et al.* 1989; Helander *et al.* 1998; Lambert *et al.* 2001; Ultee *et al.* 2002). Besides these effects on cellular membranes, essential oil active compounds (e.g. terpenes) might have several targets that inhibit bacteria involving the substrate uptake (membrane dysfunction) or electron transport (Tassou *et al.* 2000). Because of their mode of action affecting several targets, no particular resistance or adaptation to EOs has been described so far in the literature (Bakkali *et al.* 2008).

The negative correlation observed in the experiments between the bacterial concentration and larval survival was in accordance with Regunathan and Wesley (2004). The inhibition of the bacterial growth confirmed the potential of both *C. fragrans* origins to control the bacterial load in *in vivo* conditions of larval culture (Figs 1–3). This may be attributed to their antimicrobial effects as demonstrated by their low MIC against different tested isolates (Table 2). Essential oils as complex mixtures may exhibit antimicrobial activities, which differ from those of their major component tested solely (Delaquis *et al.* 2002). In fact, the inhibitory activity of an essential oil results from a complex interaction between its different constituents, which may produce, additive, synergistic or antagonistic effects, even for those present at lower concentrations (Xianfei *et al.* 2007).

Several studies have reported the potential to use plant extracts as an alternative to antibiotics in aquaculture and in organic farming in particular. Abutbul *et al.* (2004) found similar mortalities between *Streptococcus iniae*-infected tilapia, which were treated with *Rosmarinus officinalis* extract or leaf powder and those treated with oxytetracycline. Juveniles of *Penaeus indicus* fed with an enriched diets from terrestrial plants displayed better survival and growth in addition to inhibiting bacterial load of *V. parahaemolyticus* (Immanuel *et al.* 2004). The supplementation of enriched *Artemia* with plant extracts of *Withania somnifera* and *Mucuna pruriens* showed higher larval quality indices including the cumulative larval survival of *P. monodon* (Babu *et al.* 2008). The protection elicited by *Dendrilla nigra*, a marine sponge, has been reported to be because of its antibiotic effect against *V. harveyi* and *V. alginolyticus* rather than its influence on the host defence system of shrimps (Selvin and Lipton 2004).

Other studies found it unnecessary to add emulsifiers for the use of EOs (Dorman and Deans 2000; Lambert *et al.* 2001). In some cases, solvents and detergents have been reported to have an antagonist effect, which significantly decreases the antibacterial activity of EOs (Remmal *et al.* 1993). The Tween 80 has been reported to have various effects on bacteria at concentrations as low as 1, 0·5 and 0·05% (Hood *et al.* 2003) To minimize any adverse effect of Tween 80, these authors have recommended keeping its concentration below 0·05%.

This study demonstrated the potential of the essential oil of *C. fragrans* to control the bacterial load and enhance the survival rate of *P. monodon* larvae. Because of its potent antibacterial activity against *Vibrio* spp., the essential oil of *C. fragrans* could form a relevant option for developing novel antimicrobial agents for eco-friendly management of disease caused by vibrios in shrimp hatchery culture. Further studies need to be conducted to determine the specific nature of the bioactive agents of *C. fragrans* EOs.

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A Swot analysis of HACCP implementation in Madagascar

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ABSTRACT

Malagasy government is updating its legal and regulatory framework to develop a sound national food control system. In this context, promoting good hygienic practices and HACCP principles where appropriate, are important components to ensure that food safety goals are met throughout the food chain. Relevant institutions and laboratories were identified regarding four main responsibilities for which the commitment of government is vital (food policy, risk assessment, legislation, public authorities). The paper highlighted then the internal Strengths and Weaknesses, as well as the external Opportunities and Threats, the government is facing towards the implementation of GHPs and HACCP system.

1. Introduction

Foodborne microbiological risks are a growing public health concern (WHO, 2007). If not controlled, they can be a major cause of disease and premature death as well as lost of productivity and heavy economic burdens for the food sector. In Madagascar, the incidence of diarrhoeal diseases, for instance, accounted for 18% of total deaths in 2002, being the third cause of death after lower respiratory infections (27%) and malaria (22%). These diseases represented 17% of death among children under 5 years of age, similarly than the mean for the African region (WHO, 2006). Food (including drinking water) has been recognised as the major vehicle for these diseases (Motarjemi & Käferstein, 1999). In general, in developing countries, it has been suggested that both the incidence and related costs of foodborne diseases are greater than in comparable high-income countries (Henson, 2003). In fact, Madagascar is among the poorest countries with a Gross Domestic Product per capita of approximately 830 USD and 61% of the population is living below the poverty line (WHO, 2006). Foodborne microbial diseases being at the same time a cause and a consequence of poverty, the fight against these also fits within the global framework of the fight against poverty.

At international level, recent years have shown fundamental changes in the approach to food safety. The International Commission on Microbiological Specifications for Foods (ICMSF) has proposed a scheme for the management of microbial hazards for food that involves the concept of food safety objectives, FSOs, i.e., the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection (ALOP) (ICMSF, 2002). To ensure that an FSO is met, it is required to set performance objectives (POs), which correspond to the levels that must be met at earlier steps in the food chain before consumption. Control measures applied from farm to table must assure that such levels are not exceeded. FSOs and POs must be achievable by the application of good practices (GAP, GHP, GMP) and HACCP (Hazard Analysis Critical Control Point) on which, the preventive risk management strategy in the food production and industry should be based (Cordier, 2004). The implementation of food hygiene and HACCP has been reported to be an effective and cost-effective approach to food safety regulation (Crutchfield, Buzby, Roberts, & Ollinger, 1999; Mortimore, 2001; Unnevehr & Jensen, 1996). The benefits of implementing GHPs and HACCP for government, food enterprises and consumers are numerous (Table 1). For these reasons, several countries have mandated HACCP requirements into their national legislation and it becomes a crucial component of food safety in international trade (Unnevehr & Jensen, 1999).

This paper presents a Swot analysis regarding the implementation of HACCP and GHPs in Madagascar. After a presentation of factual information of the food control system in Madagascar and a

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Table 1
Benefits of GHPs and HACCP system (FAO/WHO, 2005; Unnevehr and Jensen, 1999).

Governments	Enterprises	Consumers
Improved public health Improved food security	Increased consumers and government confidence	Reduced risk of food borne diseases
More efficient and targeted food control	Reduced legal and insurance costs	Increased confidence in the food supply
Reduced public health costs	Increased market access	Improved quality of life (health, socio-economic)
Reduces costs of regulatory enforcement	Reduction in production costs	
Reduces inspection rates	Improved product consistency	
Trade facilitation	Improved staff and management commitment to food safety	
Increased confidence of the community in the food supply	Decreased business risk	

literature review on the expected role of the Government, we discuss the internal Strengths and Weaknesses, as well as the external Opportunities and Threats, the government is facing towards the implementation of GHPs and HACCP system.

The collection of information was carried out during 2007–2008 and it was based on:

- Key person interviews to obtain in-depth information from different stakeholders: leaders of food control agencies, food laboratories (public and private), involved departments of different ministries agriculture/health/economy; consumer's organisations; different international agencies based at Madagascar (Unido, EU, bilateral cooperation of France, Switzerland, Germany, USA), SMEs (street vendors, fruit processing, drinking industry, milk and cheese makers); exporters (shrimp, spices, vegetables, processed meat).
- Review of literature: available documents related to national decrees, organisational mandates and international missions reports.

2. Context of the food control system in Madagascar

According to FAO/WHO (2003), the protection of public health by reducing the risk of foodborne illness and the protection of consumers from unsafe food are among the main objectives of a national food control system. The last should cover all food produced and marketed within the country. Most systems typically comprise the five following components: (1) Food law and regulations, (2) Food control management, (3) Inspections services, (4) Laboratory services and (5) Information, education, communication and training. This publication has also underlined the importance of the integrated farm to table concept for which HACCP and good practices are fundamental and highly recommended in developing countries.

Regarding the component no. 1, a food law is under way in Madagascar with the support of the FAO (Food and Agriculture Organization) to update the food legislation. Moreover, the Office for Standards of Madagascar (BNM) (under the authority of Ministry of Economy) is responsible for drafting, centralizing and promulgating national standards (Decree No. 2004-316 on March 18, 2004).

Effective food control systems require policy and operational coordination at the national level (component no. 2). In Madagascar, food control is on the political agenda. The Malagasy Government has introduced the food safety management in the "Madagascar Action Plan" (MAP). The MAP is a five year plan (2007–2012) reflecting the eight bold commitments required to extricate the country from poverty and launch a quantum leap in medium and long-term development. Food safety management is also an objective of both the National Policy for Nutrition (PNN) and the National Action Plan for Nutrition (PNAN). They aim to "ensure the right of the entire Malagasy people to an adequate nutrition in order to improve survival of the children and to allow them a maximum development of their physical and intellectual capacities as well as promoting the health and the wellbeing of the mothers and the adults" as reported by the National Office for Nutrition (ONN, 2005) which is the authority in charge of their implementation. Another proof of the interest carried by the Malagasy Government towards the food control issue relates to the creation of the CNCA (National Committee of the Codex Alimentarius) in August 2006 (Decree No. 2006-619). CNCA is a public institution under the authority of the Ministry of Economy. It acts as an advisory body to government on matters related to Codex Alimentarius and may undertake all tasks related to food safety at the request of the Government.

Regarding food inspections (component no. 3), in Madagascar, as in many developing countries, the traditional food control programme is mainly based on establishing effective hygiene controls by end-product testing approach. These controls fall within the competence of four different public agencies: the Food Quality and Safety Control Agency (ACSQDA under authority of the Ministry of Health); the Veterinary Services of the Ministry of Agriculture and the Fishery Safety Authority (ASH under authority of the Ministry of Agriculture) which intervene, respectively in the sectors of the livestock and the fisheries; and finally the Repression of Frauds Department (SRF of Ministry of Economy). Information, education, communication and training (component no. 5 of the food control system) are part of the expected missions of these four agencies.

Finally, towards the component no. 4, the Malagasy services of inspections work in collaboration with a network of official laboratories to perform the food analyses. Those are ACSQDA laboratory, Laboratory for Chemistry and Microbiology (Ministry of Economy), National Laboratory for Veterinary Diagnostic (Ministry of Agriculture), National Research Center for Environment Laboratory (Ministry of Education).

As this article targets the implementation of GHPs and HACCP, what is their current situation in Madagascar? Only a few enterprises use HACCP system, the export sector which is harmonised with the importing countries regulation (shrimps, vanilla, litchis, spices, duck's products) and seven local enterprises which have the HACCP certification on a voluntary basis. Those are large size enterprises producing industrial food on large scale using modern equipment and tools (milk and dairy products, soft drinks, biscuits, canned fruits and vegetables). On the other hand, small and medium size enterprises (SMEs) produce a large share of the food consumed. This includes the informal sector which is made up of entities employing one or two persons including the owner (street food sector, handmade yoghourt and cheese, etc.). In general such small enterprises lack skilled personnel and knowledge of methods improving food safety. Improper food handling and storage practices, poor hygiene, limited access to safe potable water supply, poor quality of raw materials, unsuitable production environments

and problems of garbage disposal and pests are some of the many public health concerns facing this sector. Less numerous, medium size enterprises operate on more structured basis in the sector of dairy products, snacks, confisery etc. But they often encounter similar problems of poor quality raw material, lack of skills and expertise, as well as inputs, equipment and inadequate infrastructure and facilities. Poor hygienic conditions (basic sanitation, water supply, housing conditions, etc.) are a major source of contamination during production and along food processes. These medium and small local producers think that safety precautions are not worth the costs since the consumer is not willing to pay more on domestic market. The large investments and technical skills that are needed for implementation of HACCP system have economies of scale that favour larger firms (MacDonald & Crutchfield, 1996). Panisello and Quantick (2001) who investigated the technical barriers to HACCP have shown that among those barriers prior to HACCP implementation, the company size is a key reason impeding small companies due to the lack of resources, technical knowledge and incentives. So SMEs representing the highest proportion of food sector in Madagascar need assistance in order to adopt the HACCP concept and to adapt the system to their wide diversity (food processors, retailers, caterers, informal sector). Large businesses that are part of national or multinational markets are significantly more likely to implement HACCP because the higher prices consumers are willing to pay could compensate firms for the costs of food safety provisions (Mortlock, Peters, & Griffith, 1999). This is the case of the shrimp industry in Madagascar which exports to Europe.

After this description of the elements of the food control system in Madagascar, we will present a SWOT analysis for the implementation of GHPs/HACCP in the country, based on a review of the expected actions of governments in this regard.

3. SWOT analysis of HACCP and GHPs implementation

Many different actions of the role of Government may exist to enforce the implementation of GHPs and HACCP including, but not limited to, one or more of the following (Hathaway, 1999; Orriss & Whitehead, 2000; Spreij & Vapnek, 2007):

3.1. Set up and implement a national food policy

- Elaborate strategies for the implementation of GHPs and HACCP in food producing, gather information, define the obstacles and identify their causes, establish GHPs and HACCP implementation priorities relating to food types, facilities and processes.
- Create the type of environment that is conductive to promoting GHPs and HACCP by eliminating any constraint associated with their implementation and by promoting incentives means (legal, financial).
- Clarify the roles and responsibilities of the relevant agencies that are involved.

3.2. Develop scientific understanding of risks

- Develop risk assessments for a range of food/hazard combination and define food safety objectives to provide targets for the control of hazards in food by GHPs and HACCP based programmes, throughout the food chain.
- Implement risk analysis principles as a basis for decision making.

3.3. Implement legislation and enforce legal requirements

- Provide laws describing the fundamental principles of food control.
- Provide regulations prescribing the mandatory requirements and outcome to be achieved.
- Define standards and specifications that food operators' risk management programmes must meet.
- Establish sanction for non-compliance to enforce the outcome based standards.

3.4. Strengthen public authorities

- Clarify responsibilities between the different official services that are involved, establish effective communication and coordination between these services in order to implement the Codex General Principles of Food Hygiene, applicable codes of practices and relevant national food hygiene legislation by a partnership with the food operators.
- Provide a supporting role in terms of information and training of enterprises staff and inspectors.
- Set regulations for performance objectives and criteria for food operators.
- Set up unannounced inspections; validate GHPs and HACCP plans, validate industrial guides.
- Prepare generic plans to assist SMEs develop and implement their own plans for GHPs/HACCP principles. Develop adequate communication with businesses to set together those generic plans and guides to good practices using simplified documents.

According to these four principal points and in order to facilitate the evaluation of strengths and needs, as well as potential solutions, to help the Malagasy authorities to implement GHPs and HACCP among the food sector, a SWOT analysis is developed on Table 2.

SWOT analysis is a strategic planning tool consisting of two parts (FAO, 2006):

1. An analysis of the internal strengths and weaknesses: any internal asset or deficits (staff skills, equipment, finance, procedures, coordination, etc.) that enable or prevent the government from carrying out its mandate or objectives.
2. An analysis of the external environment (opportunities and threats): any external circumstance or trend (international cooperation, increased consumer awareness to food safety, global trade, etc.) that could positively or negatively affect the government's role and operations.

The four points, A, B, C and D presented in Table 2 are discussed below.

3.5. Set up and implement a national policy

It has been noticed in Section 2 that food safety management is on the political agenda of the Malagasy government. From this point of view, the promotion of modern production practices (standards and quality) and the education of all people in safe sanitary and hygienic practices are respectively mentioned as strategies to reach commitments no. 4 "rural development and a green revolution" and no. 5 "health, family planning and fight against HIV/AIDS" of the MAP. It has been mentioned as well that food safety management is an objective of both the National Policy for Nutrition (PNN) and the National Action Plan for Nutrition (PNAN). In fact, the PNN targets mainly the ponderal insufficiency of the children and malnutrition. Food safety is more an objective of the PNAN which translates into actions the PNN strategy. Thus the strategy No. 13 of the PNAN aims to improve the availability of safe food to the consumers, and to reduce the incidence of outbreaks. The principal activities related to this strategy concern: (i) development and promulgation of standards and a legislation related to food safety and food control; (ii) training and raising awareness for the application of the regulation, the standards and the certifi-

Table 2
SWOT framework related to implementation of GHPs and HACCP in food sector in Masdagascar.

Strengths	Weaknesses
Internal factors	
Implementation of a national policy	
Food safety management is on the political agenda (MAP, PNN, PNAN)	No strategy is defined for the implementation of GHPs and HACCP
	No incitation or facilities exist for food industries for domestic market to implement GHPs and HACCP
Scientific understanding of risk	
A food quality and safety control agency has been established since 2006	Food safety management is not based on risk analysis
Existence of public research centers to conduct studies	No support is given to research (risk analysis)
The national action plan for nutrition highlights the necessity to perform research on the food quality and safety	Lack of risk analysis capabilities in regulatory agencies and official laboratories
Legislation and enforcement of legal requirements	
Food law drafting is under way	Food safety is not based on food business operator responsibility
Milk sector regulations are under way in partnership with the professionals	The legislation is fragmented and out-dated
Existence of the office for standards of Madagascar to set national norms and standards	Poor control, enforcement and sanctions to assess compliance
Participation to international and regional initiatives: Codex, Comesa, Sadc	Lack of standards, specifications, directives, guidelines for food operators
Strength of public authorities	
Several agencies involved in food safety management and officially concerned with developing GHPs and HACCP among food operators	No leading infrastructure for implementation of GHPs and HACCP
Availability of competent authorities ASH (exported fisheries), Veterinary Services (products of animal origin),	Confusion and duplication in responsibilities of the various agencies involved in food safety management at central and local levels
Existence of CNCA to disseminate Codex principles and to provide a platform for discussion between different institutions	Lack of expertise, resources, budget and equipment for public agencies
Opportunities	**Threats**
External factors	
Opportunity to obtain financial and technical assistance from international donors to enhance capacities	Limited confidence of foreign governments in the quality and safety of food produced domestically
Availability of leader sectors for tourism, export and expatriate population (shrimps, litchis, strawberries, etc.)	Limited level of education of stakeholders and consumers
Availability of an accredited laboratory (LHAE)	Very limited knowledge of food hygiene among food operators and consumers
Availability of private certification organisms	Low purchase power of consumers do not encourage operators to invest into more costly quality measures (hygiene, HACCP plans, assurance quality)
Experience in conducting successful communication campaign for public health concern (vaccine)	
Existence of "Fokontany" which can act at the district level	

ASH, fishery safety authority; CNCA, national committee of codex alimentarius; Comesa, common market for eastern and Southern Africa; GHPs, good hygienic practices; HACCP, hazard analysis and critical control point; LHAE, laboratory of food hygiene and environment of institut pasteur of Madagascar; MAP, madagascar action plan; PNAN, national action plan for nutrition; PNN, national policy for nutrition; Sadc, southern african development community.

cation; (iii) setting the bodies for the quality control of food at different levels till the communes and districts; (iv) setting the bodies for supervision and monitoring of the informal food sector. To reach those objectives, a specific strategy for the implementation of hygienic practices and HACCP or any equivalent approach would be valuable. The development of this strategy and its implementation should benefit from the fact that the implementation of the PNN by ONN (National Office for Nutrition) rests on the establishment of a multisectorial coordination (sectors of health, education, agriculture and rural development, plan), from the central level (ministries) down to the Community level with the implication of the "fokontany" which are the basic administrative unit in each district. This strategy could be included in the convention that is under way between the ONN, BNM (Office for Standards of Madagascar) and CNCA (National Committee of Codex Alimentarius) to have these institutions work closely on the 13th strategy of the PNAN focussing on the "Elaboration and application of the norms and the legislation related to nutrition and food".

3.6. Scientific understanding of risk assessment and its role

At times, risk assessment is required to decide whether a FSO should be established for the hazard-food combination, i.e., the maximum frequency and/or concentration of a microbial hazard in food considered tolerable for consumer protection. This FSO can then be used by risk managers to communicate to industries this acceptable level of a hazard, in order to establish effective control measures to prevent, eliminate or reduce food safety hazards to acceptable levels using good practices and HACCP systems. As many food safety hazards can be successfully managed by using good practices and HACCP in the absence of Microbiological Risk Assessment (MRA) (ICMSF, 1997), the government should decide when a MRA may be useful and when it is probably not advisable.

Microbiological risk assessment process provides an estimate of the probability and severity of illness attributable to a pathogen-commodity combination. It requires transparent and multidisciplinary inputs from a range of food safety professionals. There are many constraints for Madagascar to contribute to risk assessment in terms of data collection, technical infrastructure, scientific and financial resources that are necessary. Since the majority of industries are not applying HACCP, they do not have a framework to share information on their risks with government agencies. Laboratory reporting diseases notification and death registration are deficient. So such inherent limitations of the database do not allow it to be used in a quantitative manner to define the magnitude of a problem.

The ACSQDA (Food Quality and Safety Control Agency), for instance, is in charge of the surveillance of foodborne diseases outbreaks in order to protect the public health. But it only records the diseases outbreaks which are declared by the hospital-based surveillance or by the medical practitioners or by the consumers directly. In case of an alert, the Agency carries out food sampling and field investigations to identify the contaminated food. In 2005 and 2006 the recorded outbreaks were up to 788 and 437, respectively (Annual Activity Report, 2006) which reflect very partially the frequency of these events as the majority of foodborne

diseases are not notified either by the consumers or by the medical practitioners.

Regarding the different official laboratories cited previously, they are equipped but face difficulties in terms of running costs, availability of reagents and media, infrastructure problems such as water and electricity cuts, material maintenance, etc. Many reasons are evoked. First of all, the demand for analyses are low in the absence of coercive measures to oblige the operators to control their products destined to domestic market. In addition, the official laboratories do not have the financial autonomy. They depend on the budgets allocated by their corresponding ministries. These budgets would be in reduction and would be sometimes versed with delays. So that laboratories are sometimes placed in situation where they cannot honour the analyses requested by the clients, which has a negative impact on their credibility.

However, Madagascar has an opportunity through the Laboratory of Food Hygiene and Environment (LHAE) of the Institut Pasteur of Madagascar (IPM) which is a private laboratory attached with the Ministry of Health. Approximately 20,000 samples are processed each year in LHAE. Since the LHAE is the only laboratory accredited according to ISO 17025 in Madagascar, it is the only one approved by the European Union for the analysis of official controls on fishery products exported to Europe. The commercial services (billing analysis, consulting and training) contribute largely to its overall operation costs. LHAE has the competence and the facilities to lead analyses within the framework of Microbiological Risk Assessments.

The international scientific cooperation is required to reinforce the role of academia and research institutions in Madagascar. Indeed, the national academic and research community lacks funds and capacities to contribute efficiently in expertise on health and food safety matters and in the identification of hazards. Actually it may play an important role in risk assessments and may serve as independent sources of information. Moreover, Madagascar should seize opportunity that the Codex Alimentarius Commission has registered ''Assistance with capacity building activities aimed at effective implementation of the principles of risk analysis" in its strategic plan for 2008–2013.

3.7. Implement legislation and enforce legal requirements

A large work is under process in Madagascar, in order to set the legal and regulatory framework for food control which is still fragmented and outdated (for instance, the law on the frauds repression dating from 1905). This project is in line with the overall strategy of the Malagasy government to promote food security, trade and economic development on a sustainable basis.

A food law is in preparation which will aim to: (i) provide the basis for the assurance of protection of human health throughout the food chain. It will establish common principles and responsibilities to underpin decision-making in matters of food safety; (ii) lay down the general principles concerning the coordination of the food controls that should address the whole supply chain and be independent and based on a sound risk analyses at national level; (iii) address all stages of production, processing, storage and distribution of food; and the import, transit and export of foodstuffs as well.

Regarding the implementation of GHPs and HACCP system in food sector, the new food law should address the following issues:

- Whether the primary responsibility for food safety will rest with the food business operators to produce safe food.
- The mandatory or voluntary application of GHPs, HACCP or any equivalent approach by industry and SMEs.
- The extent to which GHPs, HACCP or any equivalent approach would be utilized by national regulatory authorities (export commodities, domestic products, high risk food, company size).
- The development of appropriate risk analysis frameworks for decision-making on acceptable levels of safety.
- Clarify the responsibilities and relationships between involved institutions in the implementation, validation and monitoring of GHPs, HACCP or any equivalent approach.

It has been shown earlier that the BNM's missions correspond to the development, the centralization, the promulgation, the dissemination and the support of the implementation of national standards and training in standardization, certification and quality. Since 2002 (when the Board of Directors of BNM has served for the first time), the BNM has developed 28 standards related to food products but no one is related to GHPs or HACCP system application. The BNM has adopted an organizational chart which was prepared in 1999 and showed a complex structure with a target of 72 persons. In fact, the BNM has only 10 employees and lacks highly qualified staff to operate the technical committees.

3.8. Strengthen leading authorities to enforce implementation of GHPs and HACCP

In Madagascar, the four authorities implicated in food inspection, as described by Sarter (2008), would be likely to work for the implementation of GHPs and HACCP. The ACSQDA has vocation to occupy a central position in the management of food safety. Its creation's decree stipulates (Article 4) that the Agency's mission is "to protect consumer's health by ensuring that consumed, distributed, marketed or produced food in Madagascar are consistent with the highest standards of food safety and food hygiene." As such, the technical staff (18 persons) was trained as inspector of hygiene and sanitation in 2005 (funded by WHO). It is empowered to visit food establishments and restaurants to evaluate the hygienic conditions and staff awareness about good hygienic practices (approximately 500 visits in 2006). So far the inspectors of hygiene have no coercive power. They are only empowered to make recommendations. Those are based on guidelines developed by the standardization service of the ACSQDA. They are inspired from the Codex Alimentarius standards. To date, those guidelines concern the hygiene requirements for food handlers, storage, use of utensils, sale, preparation and cooking, transportation of food, structures and premises design for street catering and street food vendors. These standards may become mandatory through municipal regulations. The ACSQDA transmits its guidelines to local authorities on their request, so that they can transcribe them in their municipal regulations in the form of orders. It is the responsibility of municipal officers to conduct inspections and prepare reports in case of non compliance with these regulations.

In Madagascar, as in many countries, the official veterinary services (Ministry of Agriculture) are in charge of food safety management for products of animal origin (Act No. 2006-030 on the livestock in Madagascar). For this reason, they are in charge of food quality and hygiene inspections, elaboration of food standards and guidelines related to hygiene (Decree No. 93-844 related to hygiene and to food and animal products quality). In fact, we must distinguish between activities at central and local levels. At central level, the activities of veterinary services focus mainly on the issuance of permits and certificates (related to import and export of animal products). The other tasks and obligations that may be related to the promotion of GHPs and HACCP, such as training, increasing awareness, support for their implementation, are difficult to achieve considering the limited number of officers (three veterinary doctors and two technicians) and the lack of material resources. At the local level, controls (slaughterhouses, processing units and marketing) are under the responsibility of municipal veterinary services (Decree No. 2005-378 concerning the establishment of municipal veterinary services and establishing their

organization, allocation and operation). These are placed under the technical supervision of central veterinary services. In fact this decree is hardly applied and the control of establishments, from slaughter to retail is deficient. At slaughter level, the control is not always achieved due to the lack of qualified personnel and to the means of transportation.

The Fishery Safety Authority (ASH) is the competent authority for the control of fishery products and aquaculture in Madagascar (Decree No. 2005-375 of 22 June 2005). It operates under the authority of the Ministry of Agriculture. In fact, the activities of ASH are mainly oriented towards the control of products and establishments of fisheries and aquaculture that export to Europe. Its duties include the issuance of health certificates for fishery and aquaculture products for export and the control of hygiene practices at production, processing and storage levels of goods intended for export. Shrimps industry in Madagascar is an important socio-economic sector generating significant income for the country with a production averaging 8000 T/year (2007) that is mainly (95%) exported to Europe. This leading industry has shown that risk analysis was an integral component of the Malagasy strategy to get the approval to export to EU (European Union). An active partnership between industry and government was efficient to gain approvals and to establish a surveillance programme achieving the lowest risk by setting: (i) performance criteria and performance objectives in the processes used in this industry; (ii) the design and application of inspection, monitoring and surveillance systems for all categories of hazards (microbiological and chemical); (iii) the design and application of food hygiene systems based on GHPs and HACCP. It is worth to note that this process benefited from a support by the EU and French government assistance. This case study gave successful example for the collaboration between public authorities (Competent Authority, Ministry of Agriculture) and private companies (exporters) with the support of the international cooperation.

The Directorate of Quality and Consumer Protection (DQPC) of the Ministry of Economy is responsible for the implementation of the government policy related to quality and standardization (Decree 2007-184 of 27 February 2007 determining the functions of the Ministry of Economy). The Repression of Frauds Department (SRF) is a new department within the directorate. It is expected to be responsible for the repression of frauds in the food sector. Currently this task belongs to the Competition and Internal Trade Directorate (DCCI of Ministry of Economy as well) which operates in all sectors of commerce. To carry out this task, the new department employs only two Commissioners of Trade and three Controllers (most of the control agents are currently assigned to the DCCI). These officers are sworn and empowered to draw up penalties to sanction the contraveners with the regulation in force. Due to the lack of staff, it is clear that this mission cannot be carried out correctly. In addition, we have already noted that the legal and regulatory framework for food control is not yet operational. That is why the SRF contributes actively to the current reform of that framework (finalization of the draft of the National Food Law, drafting a new law on the repression of frauds which will replace the previous law dating from 1905, drafting a law for the protection of the consumers). Depending on the provisions that will be included in the food law related to food hygiene, the SRF could be empowered in the future to sanction non-compliance to hygiene requirements.

In conclusion, we believe that the dynamism given by updating the food legislation and regulation at a National scale, together with the opportunity given by the up-cited convention between ONN-CNCA-BNM mobilize sufficient resources to allow the country build capacity for the implementation of HACCP and GHPs. The assistance from international organisms (FAO, UNIDO, CIRAD: French Agricultural Research Centre for International Development, EU: European Union) would provide synergy to strengthen the capacities and to take decisions based on scientific evidence.

Government commitment is a vital driving force of the success of those programmes. We think that it is necessary to reinforce the role of the actual competent authorities and clarify their respective responsibilities related to hygiene implementation and monitoring in their sector (ASH/fishery; Veterinary services/products of animal origin; ACSQDA/restaurants-street vendors-imported food-processing plants). Those authorities, together with BNM will be in charge of setting the regulations and standards that businesses should satisfy. The competent authorities will develop in partnership with food businesses mentoring schemes for hygiene and HACCP plans, and will develop guides to good hygienic practices using simplified documents to assist SMEs, giving the priority to high risk food. Inspectors of the different competent authorities would be trained to be able to assess hygiene and HACCP plans. It would be worth to take advantage of the CNCA as its members gather different institutions from public and private parties to debate on how to share the responsibilities between the different stakeholders to avoid duplication and gaps in coverage. This would be an opportunity to improve the communication between the different institutions as well.

Collective actions should then be promoted to provide hygiene measures and HACCP plans where appropriate, at a larger scale than individual enterprises for SMEs that do not have in house-expertise. Guides to good practices for hygiene should be promoted too for high risk food. GHPs and HACCP are flexible enough to adapt to different firms, plants, or processes within a plant. To demonstrate their effective application, the measures outlined should be commensurate with the nature and size of the food business. Effective partnerships between government (competent authorities in particular) and food businesses are especially recommended in this regard (FAO/WHO, 2005). At level of the "Fokontany", a committee for hygiene could be created and devoted to increase awareness among food vendors, consumers, schools. This committee would be made from voluntary people that would be trained for this purpose. This organisation will provide basic instructions and training for hygiene at the base of the pyramid. Considering the challenges to implement HACCP for SMEs, and for small food businesses in particular, the priority should be given to good hygienic practices as prerequisite programmes to HACCP, providing thus a solid foundation for further development. Since microorganisms are widespread, the education of handlers on good hygienic practices is a priority. The hands of food workers play a major role in the transfer of contaminants from person to person, from person to surfaces or vice versa, and from person to food. The Recommended International Code of Practice-General Principles of Food Hygiene (CAC, 2003) is fundamental for the implementation of Good Hygienic Practices as it gives the basic rules for the hygienic handling, storage, processing, distribution and final preparation of food along the production chain. GHPs are a prerequisite programme for HACCP system which helps the food producers and processors meet Food Safety Objectives (FSOs), by identifying critical control measures that prevent, eliminate, or reduce hazards to acceptable levels.

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Composition and antimicrobial activity of essential oils of *Cinnamosma fragrans*

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ABSTRACT

Essential oil samples of *Cinnamosma fragrans* from two regions in Madagascar, Tsaramandroso (38 samples) and Mariarano (30 samples), were analysed by GC/MS. Fifty-seven components were identified, accounting from 88.3% to 99.4% of the oils' composition. The major components were linalool (72.5 ± 23.3%) in Tsaramandroso and 1,8-cineole (47.3 ± 10.2%) in Mariarano.

Samples B8 (95.8% linalool) from Tsaramandroso and B143 (71.6% 1,8-cineole) from Mariarano containing the highest proportions of the two main components identified, were selected to determine antimicrobial activities against 10 microbial strains. *Bacillus subtilis* and *Staphylococcus aureus* were the most sensitive strains to both oils. Minimum inhibitory concentration (MIC) values were lower for B143 against all tested Gram-negative strains than pure 1,8-cineole. B8 showed higher MIC values than pure linalool against *Salmonella typhimurium* and *Vibrio alginolyticus*, and similar MIC values to linalool towards the other Gram-negative strains. Both essential oils exhibited higher MIC values towards *Fusarium oxysporum* than their respective pure major component. These results suggested the occurrence of synergism or antagonism effects between the different oil constituents.

1. Introduction

Cinnamosma fragrans Baillon (local names: *Motrobeatiniana*, *Mandravasarotra*), is a tree endemic to Madagascar of the family of Cannelaceae. It is distributed extensively in the North-east of Madagascar (Canonica et al., 1969). It grows in the tropophytic forests but often near the coast. Leaves and fruits are very aromatic and have a spiced flavour (Perrier de la Bâthie, 1954). To our knowledge, little literature describes the chemical composition of this species' extracts and the information given is contradictory. For example, Schulte, Rücker, and Lewe (1972) assumed that linalool is the main compound of the essential oil of *C. fragrans* isolated from leaves, while a recent study (Tucker, Maciarello, Brown, & Griffeth, 2008) showed that 1,8-cineole and sabinene dominate.

C. fragrans is used traditionally against respiratory, parasitic and gastrointestinal infections, syphilis (Pernet & Meyer, 1957; Schulte et al., 1972), and malaria (Milijaona et al., 2003). Several essential oil components have been reported as efficient antibacterial or antifungal agents, such as linalool (Knobloch, Iberl, Weigand, & Weis, 1989), 1,8-cineole (Sökmen et al., 2004), α-terpineol, terpinen-4-ol, α-pinene, β-pinene, β-caryophyllene, α-phellandrene, *p*-cymene (Dorman & Deans, 2000). *In vitro* studies have demonstrated antibacterial activity of essential oils; *Chaenomeles speciosa* for instance, was active against several pathogens, such as *Salmonella* sp. *Escherichia coli, Bacillus sp., Staphylococcus aureus, Vibrio parahaemolyticus, Fusarium* sp., (Burt, 2004; Xianfei, Xiaoqiang, Shunying, & Guolin, 2007).

Vibrio sp. includes many strains pathogenic or opportunistic to humans, through the consumption of contaminated seafood (Sung, Li, Tsai, Ting, & Chao, 1999). *E. coli, S. typhimurium*, *S. aureus*, *B. subtilis* and *Micrococcus luteus* have been implicated in human diseases (Matasyoh, Kiplimo, Karubiu, & Hailstorks, 2007). *F. oxysporum*, which is an abundant and active saprophyte in soil and organic matter, is a plant pathogen (Nelson, 1981).

The aim of the present study was, as a first step, to determine the chemical composition of different samples of the essential oil of *C. fragrans* collected from two sites within the distribution area of the species in Madagascar. In a second step, we examined the *in vitro* antimicrobial activity of two selected samples, which

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possessed high contents of linalool or 1,8-cineole, against a large selection of Gram-negative (*S. typhimurium, Vibrio* sp., *E.* coli), Gram-positive bacteria (*M. luteus, B. subtilis, S aureus*) and one fungus (*F. oxysporum*). We compared their activities with the activity of the major component of each essential oil sample (namely pure linalool and 1,8-cineole).

2. Materials and methods

2.1. Plant material and extraction procedure

For each tree, a sample of leaves (approximately 400 g) was collected during the rainy season, in February 2005: 30 trees were harvested in Mariarano (15°41′S: 46°43′E, altitude from sea level to 7 m) and 38 in Tsaramandroso (16°18′ S; 47°02′E, altitude: 600 m) for a total of 68 samples. A voucher specimen was deposited in the herbarium of FOFIFA, Antananarivo, Madagascar. The leaves were steam distilled for 4 h in a Clevenger-type apparatus. Distillations were performed less than 24 h after sampling. The essential oils were dried over anhydrous sodium sulphate until the last traces of water were removed and then stored in dark glass bottles at 4 °C.

2.2. Chemical analysis

The 68 samples of essential oils were analysed by gas chromatography (GC) and gas chromatography combined with mass spectrometry (GC/MS).

A Varian 3400 gas chromatograph (Varian Inc., Palo Alto, CA) was used with a flame ionisation detector (FID), an on-column injector, a DB-Wax (A, J&W Scientific, Folsom, CA) fused silica capillary column (60 m × 0.32 mm i.d. × 0.25 μm film). Oven temperature was increased from 50 °C to 200 °C at a rate of 5 °C/min and held at 200 °C for 20 min. Injector and detector temperature was 230 °C. Helium was the carrier gas at a flow rate of 2.0 ml per min. Response factors were taken as 1.0 for all compounds, except for limonene (1.3), with reference to *n*-hexanol as internal standard. Linear retention indices were calculated with reference to *n*-alkanes (C_5–C_{22}). Concentrations are given as the average of triplicate analyses.

GC-MS analyses were performed under the same conditions as GC-FID using an Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass selective detector. Injector and transfer line temperatures were 220 °C and 240 °C, respectively; oven temperature programmed from 50 °C to 200 °C at 5 °C/min. Carrier gas was helium at 1 ml/min; injection volume was 0.1 μl (10% hexane solution) at a split ratio of 1:50.

The identifications of the components were based on the comparison of their mass spectra with those of Wiley and NIST (National Institute of Standards and Technology) libraries and literature data (Adams, 2001), as well as by comparison of their retention indices with literature values and co-injections.

Pure linalool and 1,8-cineole were purchased from Aldrich Chimie (Saint Quentin Fallavier, France).

2.3. Antimicrobial activity

2.3.1. Microbial strains

The activity of the essential oils samples was tested towards 10 different microorganisms. Gram-negative bacteria were *E. coli* 363, *V. anguillarum* ATCC 19264, *V. harveyi* ATCC 14126, *V. alginolyticus* ATCC 17749, *V. fisheri* ATCC 49387, *S. typhimurium* ATCC 14028. Three Gram-positive strains were *M. luteus* ATCC 10240, *B. subtilis* ATCC 6633 and *S. aureus* ATCC 6538. *F. oxysporum* ATCC 695 was the one fungus studied.

V. fisheri, *V. harveyi*, *S. aureus* and *B. subtilis* were purchased from the Collection of Institute Pasteur (Paris, France). The other strains were gifts from Ifremer (Montpellier, France). These strains were maintained on solid agar using marine agar (Difco Laboratories, Detroit, MI) for vibrios; Trypticase soy broth (Difco Laboratories) for *S. aureus, B. subtilis, E. coli*, *S. typhimurium* and *M. luteus;* Sabouraud dextrose agar (BBL, Beckton Dickinson Microbiology Systems, Franklin Lakes, NJ) for *F. oxysporum.*

2.3.2. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC were determined for samples B8 from Tsaramandroso, B143 from Mariarano, and pure linalool and 1,8-cineole as well.

A broth dilution method was used to determine the MIC and the MBC (Destoumieux, Bulet, Strub, van Dorsselaer, & Bachère, 1999). Stock solutions of essential oils were prepared in sterile distilled water. These suspensions were further diluted from 0.04 mg/ml to 23.5 mg/ml in test tubes. About 30 °C-overnight cultures were inoculated in 900 μl of Zobell (Maes & Paillard, 1992) for *Vibrio* strains or Poor broth (Destoumieux et al., 1999) for the other strains. The optical density of the inoculum was measured at D_{600} = 0.1 and then diluted to reach a final optical density of D_{600} = 0.001 in the assay. One hundred microlitres of the essential oil dilution were then added to these cultures to reach a final volume of 1 ml. The tests were carried out in triplicate. A positive control containing the bacterial culture without the essential oil and a negative control containing only the medium were analysed as well. Tubes were incubated for 24 h at 25 °C for *Vibrio sp.*, 30 °C for *M. luteus*, 37 °C for *E. coli*, *B. subtilis, S. aureus* and *F. oxysporum* (48 h incubation for the fungi).

The MIC was defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. Cell suspensions (0.1 ml) from the tubes showing no growth were subcultured on Zobell and Poor broth agar plates to determine if the inhibition was reversible or permanent. MBC was determined as the highest dilution (lowest concentration) at which no growth occurred on the agar plates (Gachkar et al., 2007).

2.4. Statistical analysis

Mean composition of essential oil was given ±SD. For comparison of MIC and MBC values, tests were made in triplicate. Analysis of variance was performed. Significant differences between means were determined by Fisher's test at the threshold of ($p < 0.05$).

3. Results and discussion

3.1. Chemical composition

The essential oils isolated from the leaves of *C. fragrans* collected in Mariarano and Tsaramandroso were characterised by their high content in 1,8-cineole and linalool, respectively (Table 1).

The samples collected in Tsaramandroso afforded essential oils with a large amount of linalool (72.5 ± 23.3%) and its oxides (1.9%); only two other oxygenated monoterpenoids accounting for more than 1% (terpinen-4-ol and 1-terpineol). The monoterpene hydrocarbons, which represented less than 10% of the mixture, were mainly represented by *p*-menthane structures α-phellandrene, β-phellandrene, *p*-cymene and terpinolene. Finally, β-cubebene, β-caryophyllene, germacrene-D, δ-cadinene and caryophyllene oxide are the main sesquiterpenoid components.

In the Mariarano plants, the oxygenated monoterpenes also represented the major chemical class. They were dominated by 1,8-cineole (47.3 ± 10.2%), the four other main components in this

Table 1
Composition of the essential oils of 68 samples (% peak area) of *C. fragrans* harvested in Tsaramandroso (n = 30) and Mariarano (n = 38).

		RI	Tsaramandroso		Mariarano		Identification method
			Mean value[A]	B8	Mean value[A]	B143	
Monoterpene hydrocarbons							
1	α-Pinene	1012	1.0 ± 1.3	0.1	3.5 ± 1.5	1.1	a; b ; c
2	Camphene	1056	1.1 ± 1.6	0.1	4.8 ± 2.2	1.2	a; b
3	β-Pinene	1097	0.9 ± 1.0	0.1	8.0 ± 3.8	2.1	a; b ; c
4	Sabinene	1113	0.9 ± 1.0	0.2	1.8 ± 0.9	3.2	a; b
5	δ-3-Carene	1142	0.5 ± 0.7	0.1	1.7 ± 1.1	0.5	a; b
6	α-Phellandrene	1157	0.2 ± 0.2	tr	0.4 ± 0.7	2.1	a; b
7	Myrcene	1160	0.2 ± 0.4	0.2	0.6 ± 1.0	1.2	a; b
8	Pseudolimonene	1167	0.3 ± 0.4	–	0.7 ± 1.3	0.2	a; b
9	α-Terpinene	1174	1.4 ± 3.7	0.2	0.9 ± 1.5	0.1	a; b
10	Limonene	1191	0.7 ± 2.3	0.2	1.3 ± 1.9	0.6	a; b
11	β-Phellandrene	1201	0.4 ± 0.6	tr	0.4 ± 0.9	0.4	a; b
12	(*Z*)-β-Ocimene	1232	0.7 ± 0.8	–	1.1 ± 1.3	0.2	a; b
13	(*E*)-β-Ocimene	1248	0.2 ± 0.3	–	0.3 ± 0.3	0.1	a; b
14	*p*-Cymene	1263	0.4 ± 1.1	0.2	0.8 ± 1.1	0.5	a; b
15	Terpinolene	1278	0.8 ± 1.4	0.1	0.6 ± 0.8	0.1	a; b
16	*allo*-Ocimene	1284	0.0 ± 0.0	tr	0.1 ± 0.3	0.1	a; b
	Total			1.5		13.7	
Oxygenated monoterpenes							
17	1,8-Cineole	1207	0.5 ± 0.9	0.4	47.3 ± 10.2	71.6	a; b ; c
18	Perillene	1294	tr	0.1	0.1 ± 0.2	tr	a; b
19	*Cis*-linalool oxide (furanoid)	1439	0.7 ± 0.9	0.1	0.5 ± 0.9	0.1	a; b
20	*Trans*-linalool oxide (furanoid)	1468	1.2 ± 2.7	0.2	0.0 ± 0.1	0.1	a; b
21	Citronellal	1472	0.1 ± 0.1	tr	0.2 ± 0.4	0.2	a; b
22	Linalool	1549	72.5 ± 23.3	95.8	1.1 ± 1.5	2.9	a; b ; c
23	Camphor	1505	0.2 ± 0.7	tr	0.2 ± 0.2	0.4	a; b
24	Bornyl acetate	1575	0.2 ± 0.4	–	0.7 ± 0.8	0.3	a; b
25	Myrtenal	1600	0.2 ± 0.4	tr	0.1 ± 0.2	0.2	a; b
26	Terpinen-4-ol	1606	1.5 ± 2.3	0.3	2.2 ± 2.0	2.5	a; b ; c
27	(*E*)-2,6 Dimethyl-3,7-octadien-2,6-diol	1669	0.3 ± 0.6	0.1	0.5 ± 1.3	–	a; b
28	α-terpinyl acetate	1687	0.1 ± 0.2	0.1	0.3 ± 0.5	0.4	a; b
29	(*Z*)-2,6 Dimethyl-3,7-octadien-2,6-diol	1696	0.0 ± 0.1	tr	0.1 ± 0.2	–	a; b
30	1-Terpineol	1700	1.2 ± 1.4	0.2	4.2 ± 3.0	2.1	a; b ; c
31	Neryl acetate	1720	0.0 ± 0.1	tr	0.2 ± 0.5	0.2	a; b
32	Geranial	1735	0.0 ± 0.1	tr	0.2 ± 0.2	0.1	a; b
33	Citronellol	1751	0.0 ± 0.1	–	0.1 ± 0.2	0.1	a; b
34	Nerol	1795	0.8 ± 1.8	–	0.5 ± 0.9	0.1	a; b
35	Isogeraniol	1811	0.1 ± 0.1	–	0.0 ± 0.1	tr	a; b
36	Geraniol	1845	0.8 ± 1.5	tr	1.2 ± 2.1	0.4	a; b
	Total			97.5		81.1	
Sesquiterpene hydrocarbons							
37	α-Cubebene	1455	0.1 ± 0.2	tr	0.2 ± 0.4	0.2	a; b
38	α-Copaene	1480	0.4 ± 1.2	–	1.4 ± 1.8	0.2	a; b
39	β-Cubebene	1535	0.9 ± 1.9	tr	0.5 ± 1.0	0.2	a; b
40	β-Caryophyllene	1654	0.7 ± 1.3	0.1	1.1 ± 1.8	0.5	a; b
41	α-Humulene	1667	0.2 ± 0.3	–	0.9 ± 1.9	0.3	a; b
42	Germacrene-D	1713	0.5 ± 0.7	–	0.7 ± 1.3	0.2	a; b
43	*Epi*-bicyclosesquiphellandrene	1730	0.0 ± 0.1	tr	0.1 ± 0.2	0.1	a; b
44	δ-Cadinene	1760	0.6 ± 1.7	–	0.5 ± 0.9	0.1	a; b
45	Cadina-1,4-diene	1783	0.6 ± 1.4	–	0.0 ± 0.2	0.2	a; b
46	*Cis*-calamenene	1853	0.3 ± 1.3	tr	0.4 ± 1.0	0.2	a; b
	Total			0.2		2.9	
Oxygenated sesquiterpenes							
47	Caryophyllene oxide	1987	0.4 ± 1.1	0.1	0.4 ± 1.1	0.4	a; b
48	Elemol	2083	0.0 ± 0.1	tr	0.0 ± 0.1	0.1	a; b
49	Nerolidol	2089	0.1 ± 0.1	tr	0.0 ± 0.1	0.1	a; b
50	γ-Eudesmol	2166	0.1 ± 0.1	tr	0.1 ± 0.1	0.2	a; b
51	Torreyol	2147	0.0 ± 0.1	tr	0.0 ± 0.0	0.1	a; b
52	Levomenol	2197	0.0 ± 0.1	0.1	0.1 ± 0.2	0.1	a; b
53	α-Eudesmol	2205	0.0 ± 0.1	tr	0.0 ± 0.1	0.1	a; b
54	β-Eudesmol	2213	0.1 ± 0.2	tr	0.1 ± 0.1	0.4	a; b
55	Driminol	2287	0.0 ± 0.1	-	0.0 ± 0.1	0.1	a; b
	Total			0.2		1.7	
Others							
56	6-Methyl-5-hepten-2-one	1279	0.4 ± 0.5	0.2	0.3 ± 0.8	tr	a; b
57	2-Hexen-1-ol	1288	0.1 ± 0.4	0.1	0.2 ± 1.0	0.1	a; b
	Total			0.3		0.1	

a: Retention index: DB-Wax.
b: GC-MS: NIST and Wiley libraries.
c: Co-injection.
tr: Trace (between 0.02% and 0.1%), compounds present below trace amounts (<0.01%) were not registered.
[A] Values are mean ± SD of 30 samples of *C. fragrans* essential oils in Tsaramandroso and 38 in Mariarano.

chemical class being 1-terpineol (4.2 ± 3.0%), terpinen-4-ol (2.2 ± 2.0%), geraniol (1.2 ± 2.1%) and linalool (1.1 ± 1.5%). The hydrocarbon monoterpenes were mainly dominated by pinenes, which represent more than 10% of the whole oil. In the group of sesquiterpenes, α-copaene (1.4 ± 1.8%), β-caryophyllene (1.1 ± 1.8%) and α-humulene (0.9 ± 1.9%) are the most abundant.

It appears that essential oils of *C. fragrans* analysed in this work could be therefore classified as "1,8-cineole type" and "linalool type". This result is close to works describing different essential oils, in which major components can constitute up to 95% of the essential oil, whereas other components are present only in trace (Bauer, Garbe, & Surburg, 2001). For example the chemical compositions of rosemary and sage essential oils were also characterised by the predominant presence of 1,8-cineole, which accounted for 88.9% and 78% of the total oils, respectively (Daferera, Ziogas, & Polissiou, 2000).

As reported in the literature, many factors such as the geographical origin, genetic factors, the plant material and the season at which the plants were collected may be responsible for the chemical composition of the essential oil (Sivropoulou et al., 1997). Andrianoelisoa et al. (2006) have described several chemotypes in *Ravensara aromatica* essential oils. Faleiro et al. (2003) have shown two major types for *Thymus* species: one rich in linalool (61.4%) and the second rich both in linalool (44.4%) and 1,8-cineole (37.4%). Chang, Chang, Chang, and Cheng (2008) have reported variations in the major components of the essential oil extracted from *Cinnamomum osmophloem* leaves amongst several regions of Taiwan. The major constituent was either *trans*-cinnamaldehyde at 91.3%, or cinnamyl acetate 56.4%, or camphor 53.7%, or linalool 95.4% depending on the region of origin. For *C. fragrans*, the essential oil composition was very different between the two sites. We can note that these two sites represent the altitudinal limits of the distribution area of *C. fragrans* (Perrier de la Bâthie, 1954).

However, we cannot conclude on the basis of this one report that the difference of the essential oil compositions between Tsaramandroso and Mariarano is of geographical and environmental origin. The hypothesis of a variation due to a genetic origin cannot be excluded (as well as that of the interaction between genotype and environment). A more detailed study is under way.

3.2. Antimicrobial activity

The MIC values ranged from 0.18 to 5.88 mg/ml for B8 (95.8% linalool) and from 0.37 to 11.8 mg/ml for B143 (71.6% 1,8-cineole) samples (Table 2). In most cases, the MBCs values of B8 and B143 were equivalent to the MIC values (bactericidal effect), except for B8 against *E. coli* and for B143 against *Salmonella, Bacillus* and *Staphylococcus* strains, for which the MBC values were higher than the MIC values (bacteriostatic effect). The main components, 1,8-cineole and linalool are well-known for their antibacterial activities (Knobloch et al., 1989; Viljoen et al., 2003). Other minor constituents have also been reported for their antimicrobial activity, such as *p*-cymene, α-pinene, β-pinene, limonene, α-terpinene, α-terpinolene, caryophyllene oxide and camphene (Sökmen et al., 2004). Our results demonstrated a higher antibacterial activity against Gram-negative bacteria for linalool, compared to 1,8-cineole, which is in agreement with other results (Faleiro et al., 2003).

Both B8 and B143 oils exhibited the lowest MIC values against two Gram-positive bacteria, *B. subtilis* and *S. aureus*. The method used to assess the antimicrobial activity, and the choice of the test microorganisms vary between publications. In most literature, Gram-positive organisms appear to be more sensitive than Gram-negative to essential oil. However, other studies did not confirm this observation, as Gram-positive bacteria have been found to be less or equally sensitive to Gram-negative bacteria as well (Burt, 2004). Our results agreed with this observation as two Gram-positive strains were the most sensitive ones whereas *Micrococcus* strain was the most resistant strain to B143 and it was as resistant as the *Salmonella* strain to B8. The permeability of the bacterial membranes, the presence of porin proteins in Gram-negative bacteria and the intracellular distribution of the oil constituents are key elements that influence the diffusion and the action of the essential oil into the cell. Variation in the essential oil activity is expected against different groups of bacteria. However, further investigations will be required to understand the mechanism of antimicrobial action of essential oils as a mixture of numerous molecules (Lambert, Skandamis, Coote, & Nychas, 2001).

Globally, *C. fragrans* samples exhibited similar MIC values against all Gram-positive bacteria to their respective pure major components, linalool and 1,8-cineole. MIC values were lower for B143 against all tested Gram-negative strains than pure 1,8-cineole. B8 showed higher MIC values than pure linalool against *S. typhimurium* and *V. alginolyticus*, and similar MIC values to linalool towards the other Gram-negative strains. Essential oil samples, tested as complex mixtures, may exhibit antimicrobial activities which differ from those of their major component tested alone (Delaquis, Stanich, Girard, & Mazza, 2002). It has been reported in the literature that the inhibitory activity of an essential oil re-

Table 2
Antimicrobial activity of B8 and B143 samples of the essential oils of *C. fragrans* from Tsaramandroso (altitude) and Mariarano (littoral) origins, respectively, and isolated linalool and 1,8-cineole.

	Linalool		1,8-Cineole		B8 Tsaramandroso (95.8% linalool)		B143 Mariarano (71.6% 1,8-cineole)	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Gram-positive								
M. luteus	5.88a	11.75b	11.75b	11.75b	5.88a	5.88a	11.75b	11.75b
B. subtilis	0.18a	0.18a	0.37b	0.73c	0.18a	0.18a	0.37b	0.73c
S. aureus	0.18a	0.18a	0.37b	0.73c	0.18a	0.18a	0.37b	0.73c
Gram-negative								
S. typhimurium	2.93a	5.88c	11.75b	11.75b	5.88c	5.88c	2.93a	5.88c
E. coli	1.47a	1.47a	2.93b	2.93b	1.47a	2.93b	1.47a	1.47a
V. fisheri	0.73a	1.47b	1.47b	1.47b	0.73a	0.73a	0.73a	0.73a
V. anguillarum	1.47a	2.93b	2.93b	2.93b	1.47a	1.47a	1.47a	1.47a
V. harveyi	1.47a	2.93b	2.93b	2.93b	1.47a	1.47a	1.47a	1.47a
V. alginolyticus	1.47a	1.47a	5.88c	5.88c	2.93b	2.93b	1.47a	1.47a
Fungi								
F. oxysporum	2.93a	ND	2.93a	ND	5.88b	ND	5.88b	ND

MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration.
Values followed by different letters within a line are significantly different by Fisher's test ($p = 0.05$).
ND: not determined.

sults from a complex interaction between its different constituents, which may produce additive, synergistic or antagonistic effects, even for those present at low concentrations (Xianfei et al., 2007). Sibanda et al. (2004), who tested the antimicrobial activity of *Heteropyxis dehniae* leaf oil (58.3% linalool as the major component) against different bacteria and fungi, found either higher, lower or similar activities to pure linalool depending on the tested microorganism. Faleiro et al. (2003) have shown that *E. coli*, which was susceptible to pure linalool, became highly resistant to a mixture containing linalool plus 1,8-cineole (1:1). Savelev, Okello, Perry, Wilkins, and Perry (2003) investigated the *in vitro* anticholinesterase activities of eight commercially available terpenoid constituents of *Salvia lavandulifolia* (1,8-cineole, camphor, α-pinene, β-pinene, borneol, caryophyllene oxide, linalool and bornyl acetate). They found a minor synergy in 1,8-cineole/α-pinene and 1,8-cineole/caryophyllene oxide combinations at higher concentrations, and an antagonistic effect in 1,8-cineole/camphor combinations with an interaction index of two.

Both essential oils exhibited higher MIC values towards the fungus *F. oxysporum* than their respective pure major component. Fraternale, Giamperi, and Ricci (2003) tested the antifungal activity of the oil of *Thymus mastichina* L, which is rich in both 1,8-cineole (55.5%) and linalool (24.5%) against different species of *Fusarium*. The MIC values varied over a range from 0.8 mg/ml to 3.2 mg/ml amongst the eight tested strains. *F. oxysporum* was not included in those selected strains, but the MIC value we found (5.88 mg/ml) was higher. However, these results might be in the same range, because our results also showed higher MIC values for pure 1,8-cineole and linalool, due probably to the different testing methods.

In conclusion, our results demonstrated that 1,8-cineole and linalool were the main constituents of essential oil samples of *C. fragrans* originating from two geographical regions in Madagascar. Both samples exhibited strong antimicrobial activity. Possible synergistic and antagonistic effects may occur in these oil samples against Gram-negative bacteria and *F. oxysporum*. On the basis of these results, essential oil of *C. fragrans* may prove to be a potentially useful antibacterial or antifungal agent.

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Decontamination of chicken skin surfaces inoculated with *Listeria innocua*, *Salmonella enteritidis* and *Campylobacter jejuni* by contact with a concentrated lactic acid solution

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Abstract 1. The aim was to establish how poultry skin could be efficiently decontaminated without changing its organoleptic properties.
2. Chicken skins were surface inoculated with *Listeria innocua* and treated with different acid solutions (2 and 10% lactic acid for 1 and 30 min). Surviving bacteria were enumerated immediately after treatment and after 7 d storage at 4°C.
3. Reductions of up to 2.6 log were reached immediately after treatment. The treatment effect persisted for 7 d storage, when the reduction exceeded 4.59 log for the strongest treatment.
4. Residual levels of lactic acid were not significantly higher than in untreated controls, except for the strongest treatment. A tasting panel found no significant difference between controls and samples.
5. After the initial results, an apparently optimal treatment (5% lactic acid for 1 min) was applied on chicken skins' surface inoculated with a mix of *Listeria innocua*, *Salmonella enteritidis* and *Campylobacter jejuni*. Treatment efficacy was assessed immediately after treatment and after 1, 4 and 7 d storage.
6. This treatment seems to be very promising from a food processing standpoint, being fast and allowing decimal reductions of 2.00 log for *Listeria innocua* and 2.38 log for *Salmonella enteritidis* after 7 d storage, neither significantly increasing skin lactic acid nor causing any organoleptic modifications to the product. The effect of the treatment is significant after one day storage for *Listeria innocua* and after 4 d storage for *Salmonella enteritidis*.

INTRODUCTION

Despite improvements by breeders and growers, and the hygiene implemented in slaughterhouses and processing plants, raw poultry carcases are heavily contaminated with bacteria (Bryan and Doyle, 1995). These bacteria often include pathogens belonging to the genera *Listeria*, *Salmonella* and *Campylobacter* (Uyttendaele *et al*., 1998; Corry and Atabay, 2001). The main site of contamination, and the one most difficult to control, is the skin. During the animal's life the skin is in permanent contact with sources of contamination and it presents numerous surface features (such as cracks and follicles) in which bacteria can be harboured (Kim *et al*., 1996). Moreover, during evisceration, further contamination of the carcase can occur. Efficient, gentle and inexpensive decontamination treatments are thus needed (Huffman, 2002). Treatments with solutions of organic acids have shown particular promise. Of the various organic acids used, lactic acid is a good candidate, as it is authorised for food products, is inexpensive, and in dilute solution has no unpleasant odour or taste. The FAO does not specify a maximum dose. The only regulations in French legislation concern cured pork products, in which a maximum of 1 g of lactic acid per kg of product is permitted (Anon, 1987). This acid occurs naturally in many

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food products, sometimes in high concentrations (at least 0·7% in yoghurt). It therefore enjoys a more ''natural'' image than chlorine, used routinely in some countries to decontaminate poultry carcases, even though studies point to the formation of carcinogenic chlorinated derivatives on contact with organic substrates (Robinson *et al*., 1981). The comparison of results obtained by different research groups is often difficult. The response of the bacteria to decontamination treatments depends on the type of organic acid used and the strain of bacteria studied. The response to decontamination is also sensitive to the way in which the bacteria are inoculated (concentration of bacteria, contact duration, temperature, conditions of humidity), their physiology, growth conditions and the substrate used (raw skin or muscle, leg or breast) (Greer and Dilts, 1992). In general, the results show that a 5-min treatment with a 1% solution of lactic acid gives an immediate reduction of the order of 0·5–1 log for *Listeria monocytogenes* inoculated on chicken skin (Gonzalez-Fandos and Dominguez, 2006). The reduction is of the order of 1 log when the concentration of lactic acid is increased to 2 or 5%. These reductions in bacterial count are low, which combined with the imprecision of the measurements (±0·1 to 0·5 log), accounts for the variability of the results reported in the literature. There are hardly any results on solutions with concentrations of lactic acid above 5%, and no systematic study has been conducted to evaluate the effect of contact time on decontamination. A persistent effect has been observed after treatment, with a slowing of bacterial growth (bacteriostatic effect) during product storage. The concentration of *L. monocytogenes* on chicken legs after 7 d of storage at 4°C, which increased by 2·94 log on untreated controls, was only 2·25 log on samples treated with 4% lactic acid for 15 s (Gonzalez-Fandos and Dominguez, 2006). During storage, the presence of indigenous flora can modify the growth dynamics of pathogenic bacteria, introducing an additional cause of variability (Gillian and O'Beirne, 1998). To the best of our knowledge, few data exist in the literature concerning lactic acid decontamination kinetics of bacteria fixed to poultry surfaces. Gonzalez-Fandos and Dominguez (2006) did not observe any persistent effect of a 5% lactic acid treatment of chicken legs surface inoculated with *Listeria monocytogenes*. After an initial bacterial reduction of 1·05 log, bacterial concentration increased similarily on the surface of untreated and treated chicken legs. However, on pork fat tissue surface-inoculated with *Listeria monocytogenes* and treated with 3% lactic acid, Greer and Dilts (1995) observed a pronounced bactericidal effect of the treatment, after one day of storage at 4°C. Decontamination kinetics vary with the decontamination treatment and the bacterial species. Using a mixture of lactic acid and sodium benzoate, Hwang and Beuchat (1995) observed a 10-fold reduction after 6 d storage for *Salmonella* spp. and 4 d storage for *Listeria monocytogenes* surface inoculated on chicken wings. No significant reduction was found for *Campylobacter jejuni*. Studies that describe the effect of treatments on the organoleptic properties of chicken products reported no significant differences in taste, colour or acceptability of pieces (upper legs or drumsticks) cooked after treatment. A slight whitening of raw products was observed by Van der Marel *et al*., 1989; Okolocha and Ellerbroek, 2005; Gonzalez-Fandos and Dominguez, 2006). None of the treatments studied used more than 5% of lactic acid. In addition, residual lactic acid residues after treatment have rarely been measured (Van der Marel *et al*., 1989). Normally, skin lactic acid concentrations are about 1·33 mg/g just after slaughter, and 2·4 mg/g after 3 d of storage. A 15-s treatment with 5% lactic acid increased this by about 2 mg/g. In addition, the diffusion of lactic acid through the skin is very slow. During treatment, Van der Marel *et al*. (1989) observed no increase in lactic acid concentration in muscle even after 3 d of storage. The measurement of the residual concentration is necessary for better understanding of the persistence of the decontamination treatments and the possible organoleptic impact on the poultry product.

The aim of our study was to determine the operating conditions that would allow efficient skin decontamination without modifying organoleptic properties. First, the effect of lactic acid treatment was assessed on *Listeria innocua*, extending it up to 10%, and investigating the effects of contact time, concentration and persistence. The *Listeria innocua* CLIP 20-595 strain was selected because its behaviour is similar to that of *Listeria monocytogenes* (Begot *et al*., 1997). It was isolated in a meat processing plant, and probably possesses a greater resistance to decontamination treatments than wild strains. In addition, many studies have shown that *Listeria* strains are more resistant to decontamination treatments than most other pathogens, in particular *Salmonella* sp. (Hwang and Beuchat, 1995). A system was developed to make inoculation more homogeneous, and tests were carried out on a homogeneous batch of chicken skins to improve the precision of results. Residual lactic acid concentration was determined and the impact of treatments on sensory properties was assessed by a panel of tasters. Thereafter, the most promising treatment from a food processing standpoint was selected, and decontamination kinetics were studied on *Listeria innocua*, *Salmonella enteritidis* and *Campylobacter jejuni*.

In order to approach real-life conditions, bacteria were inoculated as a mix, and fresh chicken skins from various batches were used.

MATERIAL AND METHODS

Inoculation

Strains of *Listeria innocua* CLIP 20-595, *Salmonella enterica* ssp. *enterica* serovar *enteritidis* CIP 82·97 and *Campylobacter jejuni* ssp. *jejuni* CIP 103726 were stored on glass beads at –18°C, to reduce as much as possible the number of subcultures and start each culture with bacteria in a similar physiological state. One bead of each of *Listeria innocua* and *Salmonella enteritidis* were placed in conical flasks containing nutrient broth (Biokar Diagnostics, France). One bead of *Campylobacter jejuni* was placed in a flask containing Preston Broth (Oxoid, France). The flasks were incubated at 37°C for 48 h for *Salmonella enteritidis* and *Campyobacter jejuni* (anaerobically), and for 24 h for *Listeria innocua*, until the growth phase was stationary. The suspensions were centrifuged at 5000 × *g* for 20 min and the pellets were resuspended in sterile saline to obtain a concentration of 7·00 ± 0·5 log cfu/ml for *Listeria innocua*, *Salmonella enteritidis* and *Campylobacter jejuni* for the decontamination kinetics trials. For the 2–10% lactic acid decontamination trials on *Listeria innocua*, the concentration of the inoculation solution was set to 8·0 ± 0·3 log cfu/ml. For the 2–10% lactic acid decontamination trials, the skins used were chicken breast skin, taken from the same batch (animals of the same age reared in the same building with the same feed) and were obtained at an industrial slaughtering facility. They were vacuum packed, deep frozen and stored at –18°C. Problems arising from wide variability of raw materials were thus avoided, enabling us to obtain reasonably reliable results with only three repetitions of each run. For the decontamination kinetics trials, in order to stay as close as possible of natural contamination conditions, inoculation was done with a mix of all three bacterial species, on raw poultry skins. Skins used came from chicken thighs (''ti'gayar'' brand, AVICOM, – ZAC des Sables, 97427 Etang Salé) bought on a daily basis at a local supermarket. In order to compensate problems arising from the wide variability of raw material coming from different batches, every assay was repeated 5 times. A disk of skin 44·6 mm in diameter was placed between a Teflon support and a stainless steel dish. Two holes drilled in the top of the dish allowed filling and draining of the dish. This laboratory-developed system was used to place the external surface of the skin in contact with 10 ml of inoculation solution. For the 2–10% trials, the *Listeria innocua* solution was placed in contact with the skin fo 2 h at 20°C. This surface was rinsed thoroughly 6 times with 25 ml of sterile saline. Untreated controls or treated samples were inoculated and rinsed in the same way. For the decontamination kinetics trials, the bacterial mix (*Listeria innocua*, *Salmonella enteritidis* and *Campylobacter jejuni*) was placed in contact with the skin for 1 min. The skins were drained for 10 min at room temperature (20°C) under a laminar flow hood, allowing adhesion of bacteria. No rinsing step was performed. Untreated controls were inoculated the same way.

Treatment

Inoculated skin was placed in contact with 25 ml of sterile saline (untreated controls) or decontamination solution (2, 5 and 10% lactic acid solution, VWR International, France) or sterile saline (control) for 1 or 30 min at room temperature (20°C). As before, after treatment the decontamination solution was removed and no rinsing was performed. The surviving bacteria on the skin were enumerated either immediately after treatment (T0), or after 1, 4 or 7 d (T1, T4 and T7) of storage at 4°C in Petri dishes closed with self-sealing plastic film. Inoculated untreated controls were examined at T0, T1, T4 and T7.

Enumeration

To enumerate the surviving bacteria, a disk 3 cm in diameter was cut out of the skin and homogenised for 5 min in 25 ml of buffered peptone water (Biokar Diagnostics, France). For the 2–10% lactic acid trials, 10-fold dilutions were made in sterile saline and immediately spread on Oxford agar (Merck KGaA, Germany) and incubated at 37°C for 24 h. For the decontamination kinetics trials, an unusual technique, derived from the Most Probable Number method (MPN) was used in order to lower as much as possible the detection threshold. Six series of decimal dilutions (from D0 to D7) were done in microwell plates (Nunc A/S, Denmark), each well containing 0·9 ml of nutrient broth (Biokar Diagnostics, France) for *Listeria innocua* and *Salmonella enteritidis*, or Preston Broth (Oxoid, France) for *Campylobacter jejuni*. Plates were incubated 24 h at 37°C (anaerobically for *Campylobacter jejuni*). For *Listeria innocua*, 5 µl of each well were dropped on Oxford agar (Biokar, France). For *Salmonella enteritidis*, 20 µl of each well were dropped on Modified Semi-solid Rappaport-Vassiliadis agar (Biokar, France). For *Campylobacter jejuni*, 5 µl of each well were dropped on Karmali agar and Butzler agar

(Oxoid, France). Petri dishes were incubated at 37°C (anaerobically for *Campylobacter jejuni*) and enumerated after 24 and 48 h. Every colony present on the selective agar matched a well considered as positive, that is, containing at least one bacterium after decimal dilutions, just as in the classical most probable number, each positive tube is supposed to have contained at least one bacterium after decimal dilution. Knowing the number of positive wells for each dilution level, it is therefore possible to calculate the bacterial concentration at the surface of the skin. As MPN tables do not cover 8-fold dilution and 6-tube cases, bacterial concentration was calculated with an MPN Calculator software (MPN Calculator build 23, Myke Curiale) and a VBA macro (Visual Basic for Applications, Microsoft, France) written for the occasion. For each sample, the bacterial concentration was expressed in cfu/cm^2, and the log of this value was used for calculations.

Experimental design

For the 2–10% trials, a two-factor two-level factorial design was chosen (lactic acid concentration of 2%, v/v, and 10%, v/v, and treatment durations of 1 and 10 min). This design enabled us to quantify the effect of the lactic acid concentration and duration of treatment and their possible interaction. Three measurements were made for each combination in the design, immediately after treatment (T0) and then after 7 d of storage (T7). In each case (T0 and T7), an inoculated and rinsed but untreated control was run, and repeated 4 times. For each combination in the design (lactic acid concentration and treatment duration), the mean log reduction was calculated from the difference between the average bacterial counts for the untreated controls (at T0 or T7) and those for the treated skin samples. Similarly, bacterial growth during storage at 4°C was calculated from the difference between the mean bacterial concentration at T7 and that at T0. The estimated repeatability for the log reduction was the deviation observed for the three measurements of average bacterial concentration. The estimated error for the bacterial growth rate corresponds to the average repeatability over all the combinations in the design. Thereafter, the most promising treatment from a food processing standpoint (5% lactic acid, 1 min) was selected for the decontamination kinetics trials. Each experimental condition (storage period of 0, 1, 4 or 7 d, treatment or untreated control) was replicated 5 times. Mean bacterial concentration and 95% confidence interval were calculated using Excel (Microsoft, France). The mean log reduction was calculated from the difference between the average bacterial counts for the untreated controls (at T0, T1, T4 or T7) and those for the treated skin samples after the same storage period (T0, T1, T4 or T7).

Determination of residual lactic acid concentrations

Extraction of lactic acid

Disks of skin 44.6 mm in diameter were treated in the same conditions as for the decontamination tests. These disks were then homogenised in liquid nitrogen. The organic acids were extracted by heating under reflux in an acid ethanol solution (20% ethanol, 0·005 mol/l HCl, VWR International, France). After centrifugation and filtration, the cations were eliminated using a cation exchange resin (Amberlite IR120). After a final filtration (0·2 µm filter), lactic acid was assayed by ion chromatography. Three repeats were run, except for the untreated controls and the 10%, 30 min treatment (5 repeats).

Assay by ionic HPLC

The assay of lactic acid was performed using a Dionex HPLC line with a precolumn (Dionex Carbopac PA1 Guard) and an ion column (Dionex Ion Pac AS11). The eluent was a water–sodium hydroxide gradient with $0{\cdot}5 \times 10^{-3}$ to 35×10^{-3} mol/l sodium hydroxide run for 25 min with a flow rate of 2 ml/min (VWR International, France). Lactic acid was quantified using a conductimetric detector (Dionex ED40), preceded by a suppressor (Dionex ASRS) to eliminate Na^+ ions by electrolysis. The standard solutions were prepared from 90% lactic acid (VWR International, France). The lactic acid concentrations were calculated from the peak areas.

Tasting

During microbiological assays, no significant colour difference was observed between treated skins and controls. Organoleptic assays relied on taste and flavour only. Tasting was carried out by a panel of 10 persons chosen at random. The sensitivity threshold of each taster was determined by having them taste, in order, solutions of increasing concentrations of acid, namely 0, 0·01, 0·05, 0·1, 0·2 and 0·5% (v/v). The sensitivity threshold of the tasters was 0·05% (6 tasters) and 0·1% (4 tasters). Disks of skin 44·6 cm in diameter were treated with solutions of 2 or 10% (v/v) lactic acid for 1 or 10 min in the same conditions as in microbiological tests. To come as close as possible to the real product (meat under skin), these disks of skin were then placed on cylinders

of filleted chicken meat of the same diameter and 1 to 1·5 cm thick. The assembly was then grilled at 180°C for 15 min. The resulting products were presented in a three-portion system. The panel were asked to taste the skin and the meat at the same time. The temperature of the products at the moment they were tasted was about 60°C. For each treatment (acid concentration – treatment duration combination), the panel tasted a treated portion and two control portions (untreated skin). They had to identify the portion that was different from the other two. When the difference was not perceptible, they made a random choice. The probability of the number of correct choices being the same as if all the tasters had chosen at random was calculated by the binomial law (10 tasters, probability of right answer for each taster = 1/3).

Statistical analysis

Statistical analysis was carried out using Statgraphics Centurion XV software (StatPoint Inc.). The significance threshold for the effects of each factor was determined by multifactor ANOVA. Before the ANOVA tests, a Levene test was carried out to check the homogeneity of the variances. To discriminate between the results of the different treatments, the least significant differences (LSD) procedure of Fisher was used with a confidence threshold of 95%. Unless otherwise stated, the uncertainties indicated correspond to the 95% confidence interval.

RESULTS

Decontamination tests on *Listeria innocua*

The results are summarised in Table 1. Table 2 presents the effects of the different factors and their significance threshold. In the case of 10% lactic acid treatment for 30 min, after 7 d storage the concentration of *L. innocua* at the surface of the skin was below the enumeration threshold in two repeats out of three. In the rest of the calculations, the bacterial concentration for this combination was arbitrarily set at the detection threshold, namely 1·67 log cfu/cm^2. In general, the efficacy of the treatments increased with the duration of treatment and the concentration of lactic acid. Immediately after the treatment the bacterial reductions were significant for all the treatments except the weakest (2%, 1 min), and for the 30-min treatments they reached respectively 1·19 and 2·66 log cfu/cm^2 with the 2 and 10% solutions. The regression coefficient of the linear model was 0·90 (cf. Table 2). The experimental data were well represented by the model chosen. The lactic acid concentration, the duration of treatment and the interaction between the two factors had significant effects on the bacterial log reduction. Over the range of values studied, the duration of treatment produced the most marked effect, and its

Table 2. *Effect and significant threshold of different factors, and equation of the* Listeria innocua *CLIP 20-595 reduction model at T0 and T7*

Factor	Log reduction	
	T0	T7
Mean	1·23	2·38
Lactic acid concentration	0·39**	1·63**
Duration of treatment	0·69***	0·42
Interaction between concentration and duration of treatment	0·34*	0·15
Regression coefficient R^2 of model	0·90	0·75
Equation of model at T0: $R = 1{\cdot}23 + 0{\cdot}39{*}C_n + 0{\cdot}69{*}D_n + 0{\cdot}34{*}C_n{*}D_n$		
Equation of model at T7: $R = 2{\cdot}38 + 1{\cdot}63{*}C_n + 0.42{*}D_n + 0{\cdot}15{*}C_n{*}D_n$		

R, log reduction; C_n, normalised lactic acid concentration (2%: −1, 10%: +1); D_n, normalised treatment duration (1 min: −1, 30 min: + 1). Significance threshold: − $P > 0{\cdot}05$; *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

Table 1. Listeria innocua *CLIP 20-595 and log reductions at T0 and T7 against duration of treatment and lactic acid concentration. Values with different superscripts in the same column (^{a,b,c}) are significantly different*

Lactic acid concentration (%, v/v)	Duration of treatment (min)	Mean bacterial concentration on the skin surface (log cfu/cm^2)		Mean log reduction (log cfu/cm^2)		Bacterial growth between T0 and T7 (log cfu/cm^2)
		T0	T7	T0	T7	
Untreated control		6·25 ± 0·15 ^{a}	6·26 ± 1·00 ^{a}	–	–	0·006
2	1	5·76 ± 0·18 ^{a,b}	5·77 ± 1·43 ^{a}	0·49	0·48	0·012
2	30	5·06 ± 0·63 ^{c}	5·23 ± 1·46 ^{a}	1·19	1·03	0·165
10	1	5·65 ± 0·32 ^{b}	2·82 ± 1·70 ^{b}	0·60	3·43	−2·824
10	30	3·59 ± 0·33 ^{d}	<1·67* ^{b}	2·66	>4·59*	−1·923

*Two of the three repeats of the 10% lactic acid treatment for 30 min after 7 d storage gave results below the detection threshold.

lengthening sharply increased the bacterial count reduction ($P < 0{\cdot}001$). The concentration of lactic acid produced an effect about half as marked as the treatment duration, but this effect was still significant ($P < 0{\cdot}01$). The positive interaction effect revealed a synergy between these two factors ($P < 0{\cdot}05$). After 7 d storage at 4°C, the uncertainty of the measurements was more marked than immediately after treatment (1·4 log cfu/cm^2 on average at T7 instead of 0·3 log cfu/cm^2 at T0). Only the treatments with 10% lactic acid gave significant bacterial count reductions. For these treatments, bactericidal action continued during storage. The bacterial concentration was lower after 7 d storage than immediately after treatment (5·62 log cfu/cm^2 at T0 against 2·82 log cfu/cm^2 at T7 for a treatment with 10% for 1 min). The uncertainty in the measurements makes it impossible to tell whether the same effect occurred with the 2% lactic acid treatments. Also, for the strongest treatment (10%, 30 min), two out of three repeats gave results below the detection threshold. The bacterial concentrations were therefore arbitrarily set at the detection threshold. It is thus impossible to tell whether a 10%, 30 min treatment was more efficacious than a 1-min treatment. The regression coefficient of the linear model was 0·75, owing to the increase in the experimental uncertainty (Table 2). Only trends can be described. The increase in the lactic acid concentration markedly and significantly amplified the reduction in the bacterial count. The duration of treatment had no significant effect, and no interaction was observed between duration of treatment and lactic acid concentration. However, this conclusion may be biased by an underevaluation of bacterial reduction in the strongest treatment – 10%, 30 min. Results below the detection threshold in two cases out of three can tell us nothing about the effect of increasing contact time on the bacterial reduction relative to the 10%, 1 min treatment.

Residual lactic acid concentrations

Residual values of lactic acid are given in Table 3. Chicken skin naturally possesses a high lactic acid content (90 µg/cm^2). We observed an apparent effect of lactic acid concentration and duration of treatment on the residual lactic acid concentration, and a marked interaction of these two factors. However, the wide variability of the results did not allow us to differentiate the residual values in the treated samples and the untreated controls, except for the strongest treatment (10%, 30 min), for which it was more than 7 times higher than the control. The uncertainty for this last item is marked, but this uncertainty increased linearly with mean residual concentration: the relative uncertainty represents 36–50% of this residual concentration.

Organoleptic tests

Of the 10 tasters, 4 detected lactic acid in solution from a threshold concentration of 0·1% (v/v) and 6 from a concentration of 0·05%. The results obtained in tasting sessions are given in Table 4. For all treatments the *P* value was much higher than the significance threshold (0·05). The proportion of correct choices was therefore not significantly different from what would have been expected if all the tasters had answered randomly (0·33). No significant difference in taste was thus found for any of the treatments.

Acid decontamination kinetics

Figures 1, 2 and 3 show the changes in average concentration of *Listeria innocua*, *Salmonella enteritidis* and *Campylobacter jejuni* respectively at the surface of untreated controls and after a 5% lactic acid treatment for 1 min, immediately after treatment (T0) and after 1, 4 and 7 d storage at 4°C (T1, T4 and T7, respectively). Immediately after treatment, bacterial concentration at the surface of skin samples was not significantly different between untreated conrols and treated skins, for the three tested bacteria. Concentrations of *Listeria innocua* on treated skins were significantly lower than on untreated

Table 3. *Residual lactic acid on skin after different decontamination treatments*

Treatment			
Concentration (%, v/v)	Duration (min)	Mean lactic acid concentration on skin (µg/cm^2)	i.c. 95% (µg/cm^2)
0	0	90[a]	45
2	1	112[a]	48
2	30	122[a]	45
10	1	165[a]	62
10	30	731[b]	337

Values with different superscripts ([a,b,c]) are significantly different.

Table 4. *Number of tasters who correctly distinguished the treated sample from the two controls. The* p *value reflects the probability that the choice was not statistically random*

Treatment	Number of correct choices (out of 10 tasters)	*p* value
2% 1 min	4	0·88
2% 30 min	2	0·60
10% 1 min	4	0·88
10% 30 min	5	0·43

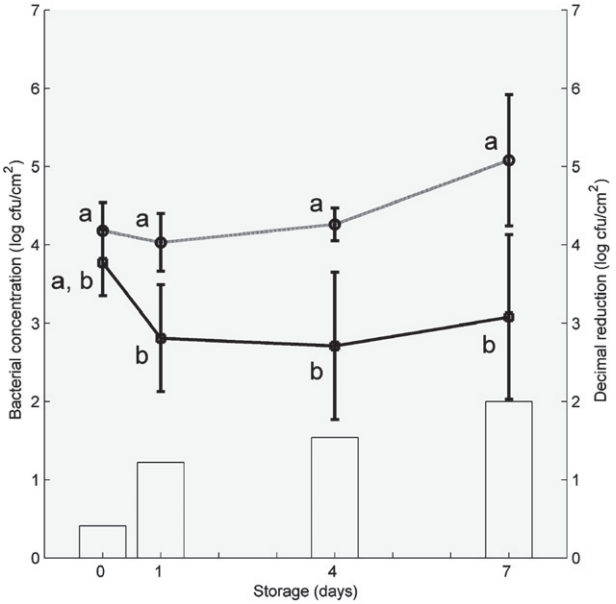

Figure 1. Listeria innocua *CLIP 20-595 average concentration on the surface of untreated controls (circles, dotted line) and on skins treated with a 5% lactic acid solution for 1 min (squares, full line) and decimal reductions (bars), for different storage periods at 4°C. Error bars: confidence interval at 95%. Values with different letters (a, b) are significantly different.*

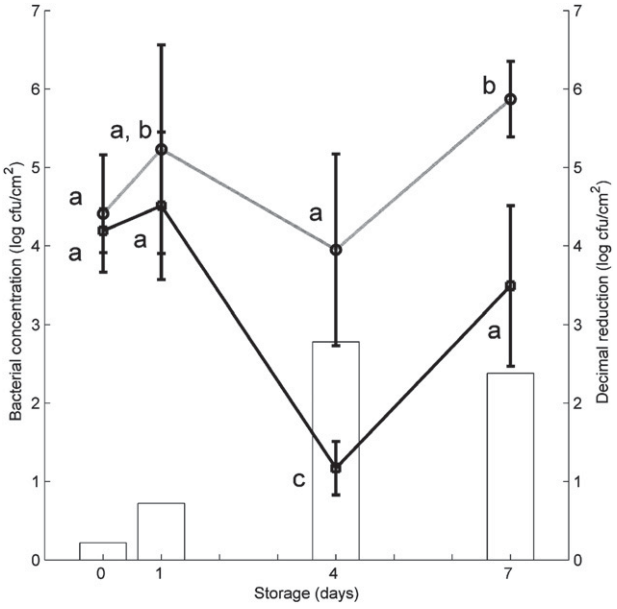

Figure 2. Salmonella enteritidis *CIP 82·97 average concentration on the surface of untreated controls (circles, dotted line) and on skins treated with a 5% lactic acid solution for 1 min (squares, full line) and decimal reductions (bars), for different storage periods at 4°C. Error bars: confidence interval at 95%. Values with different letters (a, b, c) are significantly different.*

samples from one day of storage onwards. Decimal reduction reached 1·22 log cfu/cm^2 and increased during all the storage period, finally reaching 2·00 log cfu/cm^2 after 7 d storage. A significant difference between *Salmonella enteritidis* concentrations on treated skin and

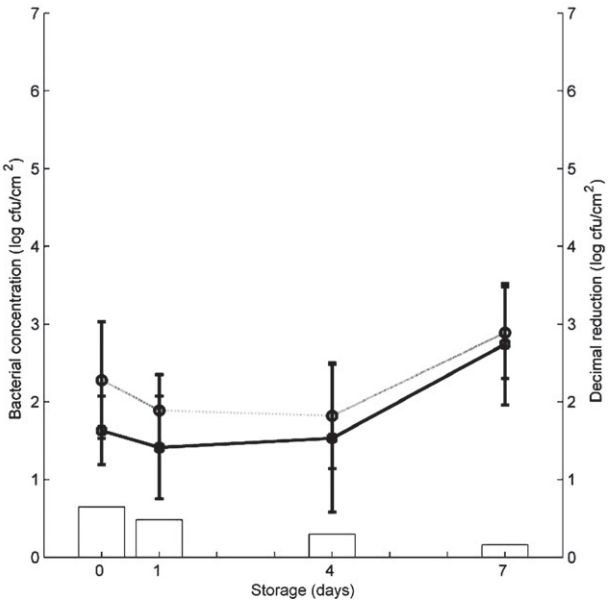

Figure 3. Campylobacter jejuni *CIP 103726 average concentration on the surface of untreated controls (circles, dotted line) and on skins treated with a 5% lactic acid solution for 1 min (squares, full line) and decimal reductions (bars), for different storage periods at 4°C. Error bars: confidence interval at 95%. There was no significant statistical difference between treated and control groups.*

untreated controls could be observed from 4 d storage onwards. Decimal reduction reached 2·78 log cfu/cm^2 and remained constant up to 7 d of storage. For *Campylobacter jejuni*, average bacterial concentration on the surface of treated skins was lower than on untreated controls. However, great variability combined with the low inoculation values made it impossible to detect significant bacterial reductions between treated skins and untreated controls.

DISCUSSION

Lactic acid decontamination of *Listeria innocua*

The inoculation system used allowed an even surface treatment and accurate control of the inoculated and treated surface. The rinsing step left only bacteria that were strongly adherent or harboured in surface irregularities such as folds, cracks and follicles, and which are therefore likely to be more resistant to decontamination treatments. It also made it possible to limit the resuspension of the bacteria when the decontamination treatment is applied, which would lead to an overestimation of the efficacy of the treatment. Immediately after the treatment, the bacterial reductions obtained with the 2 and 10% solutions were greater than those observed by Gonzalez-Fandos and Dominguez (2006), who for treatments lasting 5 min with 1·6 and 4%

obtained reductions of 0·6 and 1·05 log, respectively. However, these authors worked with *Listeria monocytogenes*, and used chicken legs, whereas we worked on breast skin. Capita *et al*. (2002) showed that the place where the skin was sampled (breast, legs or back) affected the observed efficacy of trisodium phosphate decontamination treatment. This effect is linked to both the way the active substance diffuses in the skin and the unevenness of the surface (cracks, folds, follicles), and its fat content, which can protect bacteria from the treatment. The same effects may occur for lactic acid treatments. The abdominal skin of the chicken used in our experiments was very thin and lean compared with the leg skin. It is therefore probable that the efficacy of the decontamination was slightly greater in our study than that observed by Gonzalez-Fandos and Dominguez (2006). Because the results obtained with a lactic acid concentration of 10% are new, no comparison with the literature is possible. After 7 d of storage at 4°C, the increased uncertainty of the measurements may have two main causes. First, an additional storage step brought a further cause of variability, even if all storage variables (temperature, humidity) were as close as possible for all the samples. Second, the growth and death of bacteria during storage may be under the combined influence of factors such as the concentration of bacteria on the skin surface at the end of treatment, the structure of the skin and the residual level of lactic acid. Thus, the variations of these factors could cause variations in the concentration of bacteria on the skin at the time of analysis, especially after long storage times. After storage, only the treatments with 10% lactic acid gave significant bacterial count reductions. Gonzalez-Fandos and Dominguez (2006) observed more marked differences between treated samples and controls after 7 d storage compared with T0. However, these differences remained limited and were observed in a rapidly growing bacterial population. In the experiments of Gonzalez-Fandos and Dominguez (2006), the population of *Listeria monocytogenes* increased by 2·9 log in 7 d in controls and by 2·2 log in samples treated with a 5% acid solution for 5 min. The effect of the acids was thus a moderate slowing of growth during storage. By contrast, in our study no bacterial growth was observed, even in untreated controls, and a strong bactericidal effect was observed with 10% acid solutions. Our numbers of *L. innocua* after 7 d storage were thus completely different from those of Gonzalez-Fandos and Dominguez (2006). The concentration measured by these authors was 5·95 log for a treatment with 5% acid, against less than 1·67 log with our strongest treatment. Different hypotheses can be advanced to explain why the population of *L. monocytogenes* continued to grow in the experiments of Gonzalez-Fandos and Dominguez (2006) whereas no growth was observed in our study. The number of *Listeria* inoculated were much lower in Gonzalez-Fandos and Dominguez (4·27 log) than here (6·26 log). This can be explained by the differences in inoculum contact time: 5 min for Gonzalez-Fandos and Dominguez (2006) against 2 h for us. Short contact times may favour the selective adhesion of physiologically more active bacteria that are thus better able to grow afterwards. The fact that the bacteria are more numerous may cause more intense competition for the substrate, or different growth behaviours usually described as the Jameson effect, which refers to the suppression of growth of all microorganisms by high total microbial loads (Ross *et al*., 2000; Gimenez and Dalgaard, 2004). The differences may also be explained by differences related to the bacterial strain, substrate or relative humidity of the air surrounding the product during its storage (Hills *et al*., 2001; Beales, 2004).

Residual lactic acid

The residual concentrations of lactic acid were not significantly different from those of untreated skin samples, except for the strongest treatment (10%, 30 min). These results differ from those of Van der Marel *et al*. (1989), who observed a significant difference in residual concentrations between controls and upper leg skin treated for 15 s with a 2 or 5% solution of acid (1·98 mg/g for the sample treated with 2% lactic acid against 1·33 mg/g for the control). These differences may be due to the use of a specific measurement method inducing lower uncertainties, or a greater irregularity of the skin (splits, cracks, follicles) in this earlier work, causing a greater absorption of lactic acid. Differences in fat content may also modify the rate of diffusion of acid in the skin. The values of residual lactic acid measured here account for the results obtained in the microbiological tests for the strongest treatment (bacterial reduction greater than 4·59 log cfu/cm^2 for 10%, 30-min treatments). The high lactic acid residue probably continued to destroy bacteria during storage, which explains why the bacterial concentration was less than 1·67 log cfu/cm^2 after 7 d of storage. For the 10% and 1-min treatment, however, the residual lactic acid was not significantly different from that measured in the control, although bacterial destruction also continued during storage. This may be because the bacteria were concentrated at the skin surface. The residual lactic acid was measured in the whole thickness of the skin and probably

underestimated the residual lactic acid actually in contact with the bacteria. No measurement of the coefficient of diffusion of lactic acid in skin could be found in the literature. However, after the treatment of upper legs with 5% lactic acid for 15 s and 3 d of storage, Van der Marel *et al*. (1989) observed a significant increase in the lactic acid in the skin but not in underlying muscle, consistent with a slow diffusion of lactic acid in the skin. It might be useful to develop a diffusion model to predict residual values according to duration of treatment and concentration of decontaminant solution. However, it would seem more relevant from a food processing standpoint to emphasise short treatment times, whereby the product is decontaminated leaving non-perceptible lactic acid values close to those naturally occurring on the skin.

Organoleptic tests

To our knowledge such a broad range of experimental conditions has not been studied before. However, for the gentle treatments our results are consistent with those of Okolocha and Ellerbroek (2005), where a tasting panel found chicken carcases sprayed with 1% lactic acid for 10 s and then grilled to be acceptable to very acceptable. They also agree with those of Van der Marel *et al*. (1989), where the tasting panel gave no preference between controls and upper legs treated for 15 s with 1% lactic acid solutions, and found no off-flavour, although whitening was observed on raw samples. The lack of any difference in taste even with the strongest treatments confirms that lactic acid is a good candidate for decontaminating poultry surfaces.

Acid decontamination kinetics

Listeria innocua decimal reduction values fit well between those observed during treatments of same duration, with lactic acid solutions at 2 and 10% (Table 1), whether immediately after treatment or after 7 d storage, in spite of the differences in methods and inoculation. No significant growth was observed on untreated controls during storage, by contrast with the 2·9 log increase of the *Listeria monocytogenes* population observed by Gonzalez-Fandos and Dominguez (2006). The extent of inoculation of *Listeria* being similar, due to the short contact time (4·27 log in Gonzalez-Fando and Dominguez, 2006 and 4·18 log cfu/cm^2 here), the only remaining hypothesis explaining such absence of growth are differences related to the bacterial strain, substrate or relative humidity of the air surrounding the product during its storage (Hills *et al*., 2001; Beales, 2004). On untreated controls, *Salmonella enteritidis* concentrations increased significantly during storage, to the same extent as observed by del Rio *et al*. (2006). With regard to *Listeria innocua* and *Salmonella enteritidis*, the effect of treatment was not obvious immediately, but became significant from 1 and 4 d of storage at 4°C, respectively. After 4 d storage, *Salmonella enteritidis* concentration decreased significantly on treated samples. Between 4 and 7 d, the *Salmonella enteritidis* concentration increased at the same rate on treated samples and untreated controls. Such a pattern could be explained by the effect of the longer lag phase of *Salmonella enteritidis*, observed during preliminary growth kinetics on nutrient broth, on the competition between bacterial growth and bacterial death. Until 4 d of storage, bacterial reduction was more important than bacterial growth. After 4 d, the lag phase being over, bacterial cells could increase exponentially, and bacterial growth was more important than bacterial death. A similar pattern could be observed for *Listeria innocua*, whose lag phase is shorter. The treatment induced bacterial death after one day of storage. Between 1 and 7 d, there was no significant increase of the *Listeria innocua* concentration, whether on treated samples or untreated controls. After 7 d storage, the bacterial reductions of these species were almost identical (respectively, 2·00 and 2·38 log cfu/cm^2). In contrast, the decontamination treatment did not produce a significant effect on *Campylobacter jejuni*, although no significant increase of the bacterial concentration could be observed even after 7 d storage. To the best of our knowledge, there are no data on the decontamination kinetics of *Listeria* spp., *Salmonella* spp. or *Campylobacter* spp. on poultry surfaces with lactic acid solutions. The decontamination kinetics observed in our study are closer to those observed by Greer and Dilts (1992) during lactic acid decontamination of *Listeria monocytogenes* inoculated on pork fat tissue than those observed by Hwang and Beuchat (1995) during the decontamination of *Salmonella* spp., *Listeria monocytogenes* and *Campylobacter jejuni* surface inoculated on chicken wings, using a lactic acid/sodium benzoate solution.

Thanks to our initial results, the most promising treatment from a food processing standpoint was chosen. A 5% lactic acid treatment 1 min long is fast, allowed log reductions in *Listeria innocua* and *Salmonella enteritidis* counts of 2·00 and 2·78 log cfu/cm^{-2} after 7 d of storage, did not significantly increase the lactic acid in the skin (and so even less in the whole product) and therefore did not cause any organoleptic modifications to the product. The decontamination kinetics during storage differed between bacterial species, the efficacy of the treatment appearing significant earlier in the case of

Listeria innocua (one day storage) than in the case of *Salmonella enteritidis* (4 d storage). Although the efficacy of such treatment has been confirmed on *Salmonella enteritidis* and *Listeria innocua*, those results need to be confirmed on the other pathogen commonly found on chickens, *Campylobacter jejuni*. New methods should be developed to reduce the variability of enumeration with increased numbers of *Campylobacter jejuni* during the inoculation step. Moreover, the treatments should be tested on a plant scale, on naturally contaminated carcases, as the response of inoculated bacteria to decontamination treatments is not necessarily the same as the indigenous flora. Looking forward, this acid treatment could be readily combined with steam treatment, which would afford more marked immediate bacterial reductions, but without a persistent effect (James *et al*., 2000; Logue *et al.*, 2005). Such combined treatment should allow more efficacious and longer-lasting decontamination than either of the treatments used alone. The rate of diffusion of lactic acid in skin is not known, but a useful and persistent effect has been demonstrated. The systematic measurement of the residual lactic acid at different contact times and acid concentrations could make it possible to obtain optimal conditions in which the surface bactericidal effect was maximal, while at the same time ensuring residues below the threshold of acidity in taste perceptible by consumers. However, this research would require more precise measurement of residual acid quantities. Lactic acid treatments should therefore make it possible to improve the microbial safety of chicken without adversely affecting its sensory characteristics.

Nevertheless, the use of antimicrobial chemicals is still not allowed in the European Community for poultry decontamination. However, this point is under debate and a draft of regulations have been proposed which does not include lactic acid as an authorised antimicrobial chemical and imposes rinsing off of the product after treatment. We consider it a mistake not to consider lactic acid as an antimicrobial. Lactic acid is a natural component of poultry skin which is not detrimental to human health at antimicrobial concentrations, these concentrations being not significantly increased by the treatments proposed in this work. The mandatory rinsing off of the product is due to the distinction between ''processing aid'' and ''preservation'' which is important from a general regulation point of view but not really relevant from a scientific point of view for this type of natural chemical at the suggested concentrations.

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Effects of mycotoxins, aflatoxin B_1 and deoxynivalenol, on the bioluminescence of *Vibrio fischeri*

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Abstract

The effects of aflatoxin B_1 and deoxynivalenol on the luminescence of *Vibrio fischeri* were investigated to determine the conditions of using the bioluminescence as an indirect means for mycotoxin detection. The culture of *Vibrio fischeri* showed that bioluminescence reached a peak after 12 hours of incubation at 25 °C and then decreased drastically. During the lag phase which lasted 6 hours, light emission decreased drastically for both the mycotoxin assays – aflatoxin B_1 10 µg/ml and deoxynivalenol 20 µg/ml – and the corresponding controls. Distinct bioluminescence inhibition appeared after this period of minimal bioluminescence of the controls and started with the exponential phase of growth. The percentage of bioluminescence inhibition for both mycotoxins was determined after 3.5, 10, 15 and 25 hours of incubation. The bioluminescence of *Vibrio fischeri* was inhibited with aflatoxin B_1 and enhanced with deoxynivalenol. Both effects were delayed and required a long-term incubation over 10 hours, which may help to investigate bioassays for mycotoxin detection.

Keywords: toxicity, microorganism, control methods

1. Introduction

The prevention of mycotoxin contamination of food and feedstuffs is one of the top priorities in human and animal safety. Mycotoxins are secondary fungal metabolites that have been shown to be carcinogenic, mutagenic, teratogenic and immunosuppressive (Enomoto and Saito, 1972). Among the most considered mycotoxins are aflatoxins (and mainly B_1) produced by *Aspergillus* spp. and deoxynivalenol (DON) produced by *Fusarium* spp. These contaminate many commodities such as cereals and grains, peanuts and spices (Gatt *et al.*, 2003; Gonzalez *et al.*, 1999; Sarwar Nasir and Jolley, 2002; Vandeven *et al.*, 2002; Yumbe-Guevara *et al.*, 2003). Toxicity bioassays using the inhibition of bacterial luminescence are currently used in ecotoxicology and environmental technology (Fernandez-Alba *et al.*, 2002; Peinado *et al.*, 2002; Repetto *et al.*, 2001; Ribo and Kaiser, 1983). The application of the luminescence-based bioassays shows a great potential for determination of contaminants in food and feed. In the specific case of mycotoxins, the application of those bioassays might help as alternative, low cost and reliable screening tools for indirect mycotoxin detection (qualitative approach), in comparison to the classical sophisticated and expensive chromatographic methods (Sarter and Zakhia, 2004). However, very little literature has reported the application of these bioassays for the determination of mycotoxins (Yates and Porter, 1982). The purpose of this study was to investigate the effect of aflatoxin B_1 and DON on the luminescence of *Vibrio fischeri* and to discuss essential parameters that could help setting an innovative bioassay for mycotoxins.

2. Material and methods

The bioluminescent marine bacteria *Vibrio fischeri* (CIP 105356) was purchased from the Pasteur Institute Collection (Paris-France). Stock cultures of the microorganism were maintained on Bactomarine agar (Difco, Detroit, USA) at

4°C. Liquid cultures were performed in Erlenmeyer flasks each containing 50 ml of Marine broth (Difco, Detroit, USA). These cultures were inoculated with 24 h-colonies from Bactomarine agar Petri dish and then incubated in a shaking water bath at 25 °C at 30 rpm.

Cell growth was assessed by absorbance at 550 nm using a spectrophotometer (Milton Roy Spectronic 1201). Bioluminescence was measured with a luminometer Lumat (Berthold, Germany). Bacterial absorbance at t_0 (time zero) was adjusted to 0.1 at 550 nm (corresponding to 10^6 cfu/ml). Bacterial suspensions were vortexed to ensure uniformity and luminescence was measured immediately after vortexing.

DON and aflatoxin B_1 were purchased from Sigma Aldrich (respective references A6636 and D0156). For the mycotoxin assays, DON was dissolved in methanol and aflatoxin B_1 in DMSO (dimethyl sulfoxide). Tests with mycotoxins (500 µg aflatoxin B_1 and 1000 µg DON were dissolved in 500 µl of DMSO and methanol respectively, and then filled up to 50 ml with culture to achieve final concentrations of 10 µg/ml aflatoxin B_1 and 20 µg/ml DON respectively) were conducted in triplicate using nominal concentrations for mycotoxins. Controls without mycotoxin, containing the culture plus 500 µl of the corresponding solvent, methanol or DMSO, were run in triplicate simultaneously for each mycotoxin assay.

The percentage of bioluminescence inhibition was determined after 3.5, 10, 15 and 25 h of incubation according to Froehner *et al.* (2002) using the following formula: Inhibition (%) = $((C_{t10} - S_{t10}) 100) / C_{t10}$ where C_{t10} specifies the arithmetic mean of the bioluminescence values of the controls after 10h and S_{t10} indicates the bioluminescence value of a particular sample after 10h (idem for $t_{3.5}$, t_{15} and t_{25})

3. Results

The culture of *Vibrio fischeri* over 25 h showed that bioluminescence reached a peak after 12 hours of incubation (Figure 1). During the lag phase which lasted 6 hours as shown on the growth curves of *Vibrio fischeri* cultures (Figure 2), the light emission decreased drastically for all cultures, either mycotoxin assays and the corresponding controls (Figure 3). After 10 hours of incubation, the bioluminescence of the DON assay exceeded the control bioluminescence, but remained zero for the aflatoxin assay (Figure 4). The percentage of bioluminescence inhibition for both mycotoxins (10 µg/ml aflatoxin B_1, 20 µg/ml DON) was determined after 3.5, 10, 15 and 25 h of incubation (Figure 5). Aflatoxin B_1 inhibited totally the bioluminescence of *Vibrio fischeri*, while DON enhanced it after the lag phase (Figure 5).

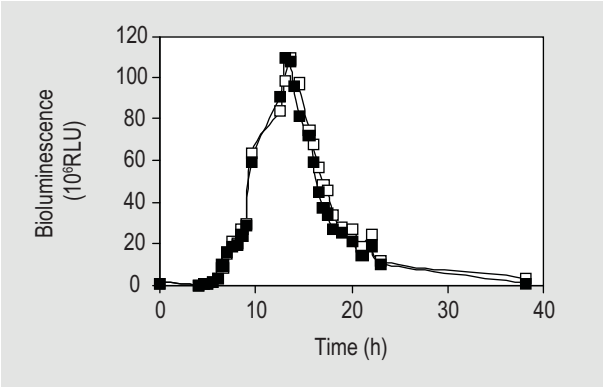

Figure 1. Evolution of *Vibrio fischeri* bioluminescence during the lifecycle over 30 h of incubation (in duplicate). RLU: relative luminescence units.

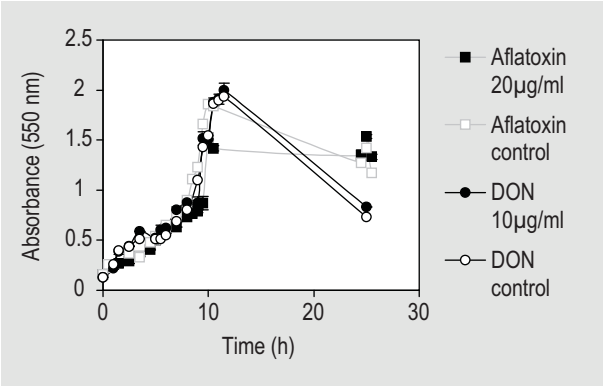

Figure 2. *Vibrio fischeri* growth curve for mycotoxin assays (10 µg/ml aflatoxin B_1 and 20 µg/ml DON) and the corresponding controls.

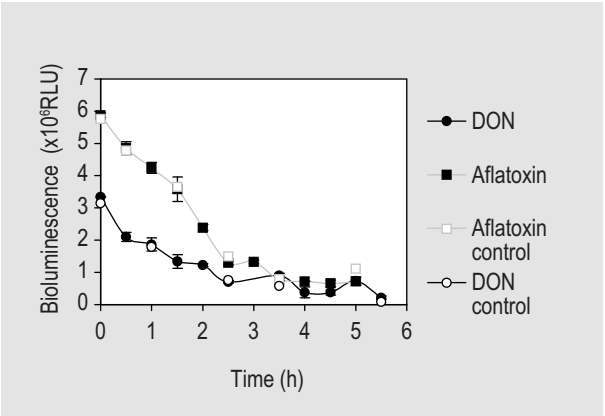

Figure 3. Evolution of *Vibrio fischeri* bioluminescence during the lag phase of growth for mycotoxin assays (10 µg/ml aflatoxin B_1 and 20 µg/ml DON in triplicate) and the corresponding controls (in triplicate). RLU: relative luminescence units.

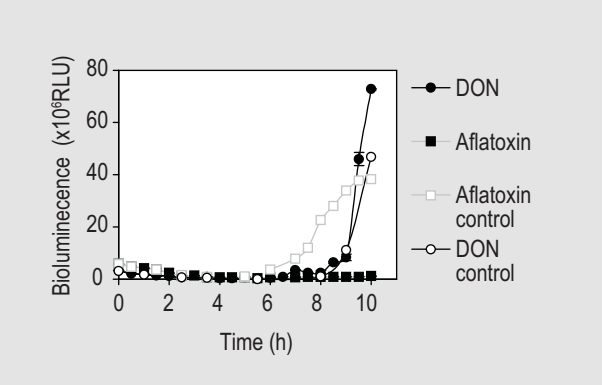

Figure 4. Evolution of *Vibrio fischeri* bioluminescence during the first 10 hours of growth for mycotoxin assays (10 µg/ml aflatoxin B_1 and 20 µg/ml DON in triplicate) and the corresponding controls (in triplicate). RLU: relative luminescence units.

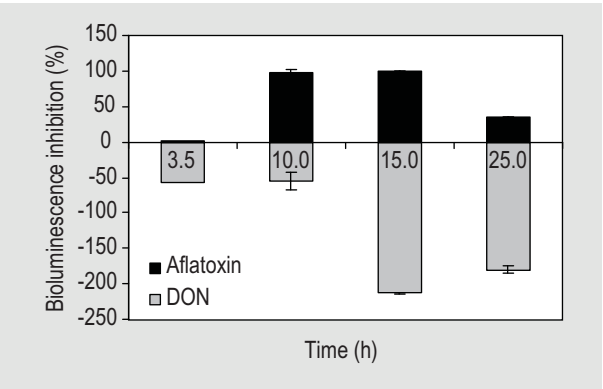

Figure 5. Percentage of bioluminescence inhibition after 3.5, 10, 15 and 25 h of incubation for mycotoxin assays (aflatoxin B_1 10 µg/ml and DON 20 µg/ml in triplicate) using *Vibrio fischeri* at an absorbance of A_{550}=0.1 A_{550}: absorbance at 550 nm.

4. Discussion

These results showed that the bioluminescence inhibition clearly appeared after the period of minimal bioluminescence of the controls which corresponded to the lag phase of growth. As the expression of bioluminescence genes is induced by two quorum-sensing systems, *ain* and *lux* in *Vibrio fischeri*, intracellular responses are synchronized with the population density which make the bacteria emit light effectively only when they are at high cell density (Lupp *et al.*, 2003; Lupp and Ruby, 2004). In these conditions, it would not have been possible to detect any mycotoxin effect during the first 6 hours of incubation (lag phase). From this point of view, a long-term incubation, over 10 hours, was required for the evaluation of the mycotoxin impact on bacterial bioluminescence.

Similar behaviour was reported by Froehner *et al.* (2002) who described a decrease in the *Vibrio fischeri* bioluminescence in the first 12 hours of incubation while the cells were growing. The bioluminescence then rose constantly from 12 h to 24 h of incubation. This time interval is longer than ours, probably due to the difference in the temperature of incubation since these authors worked at 15 °C. Since the bioluminescence decreased in the first hours of incubation, a delayed toxicity is difficult to detect with short-term tests. Backhaus *et al.* (1997) have demonstrated that the toxicity of certain chemicals, mainly antibiotics, was underestimated by a short-term assay (30 min) carried out according to the ISO 11348 (ISO, 1998) but not by the 24 h long-term bioluminescence assay with *Vibrio fischeri*. These authors found an important increase in toxicity with prolonged exposure time for specifically acting chemicals (group 1: chloramphenicol, nalidixic acid, tetracycline) but not for unspecifically acting chemicals (group 2: sodium dichromate, 3,4-dichloroaniline, cetyltrimethylammonium bromide). Froehner *et al.* (2000) who tested different incubation periods (30 min, 7 hours and 24 hours) concluded that substances causing acute toxicity like pentachlorophenol, 3,4 dichloroaniline and dodecylpyridiniumbromide could be assessed with an equal sensitivity in short-term assays (30 min of incubation) as well as in long-term assays (7 h and 24 h of incubation). They emphasized that the increased sensitivity of long-term assays (24 h and 7 h tested) for substances with delayed toxicity (nalidixic acid, chloramphenicol and streptomycinsulphate) could be ascribed to effects on biosynthetic processes like protein- or DNA-biosynthesis which keep the bacteria vital, luminescent and alive over 24 h. On the other hand, effects after 30 minutes mainly reflect an interference with the energy status of the cell.

According to this literature, we can assume that the impact we observed for aflatoxin B_1 in our experimental conditions is a delayed toxicity rather than an acute one. Further investigations on the mechanisms of action of this mycotoxin on bacteria should provide better understanding.

Most literature exist for chemicals, antibiotics and water contaminants; some literature deals with mycotoxin bioassays. Yates and Porter (1982) adapted the Microtox analyser (Beckman Instruments) used for aquatic toxicity to mycotoxins. They reported an inhibition of *Vibrio fischeri* bioluminescence by different mycotoxins after 5 min of incubation and EC50 (mycotoxin concentration that reduces the bioluminescence by 50%) ranged from 31.79 µg/ml for rubratoxin B to 7.53 µg/ml for patulin. The EC50 for aflatoxin B_1 was 21.97 µg/ml after 5 min and 20.35 µg/ml after 20min. This short-term assay might be due to the different experimental conditions specifically used in the patented Microtox analyser (Bulich and Isenberg, 1981; Park and Hee, 2001; Yates and Porter, 1984).

The DON assay (10 µg/ml) showed a hormesis effect since the bioluminescence surpassed the bioluminescence of the control. It has been reported that bacterial luminescence can be enhanced under stress conditions that cause DNA damage. Weiser *et al*. (1981) have shown that physicochemical agents that interact with DNA can revert the dark mutants of *Photobacterium leiognathi* to genetically stable luminous cells. Czyz *et al*. (2002) have reported stimulation of bioluminescence of *V. fischeri* four hours after the addition of different mutagen agents at different concentrations (2-aminofluorene, 4-nitro-*o*-phenylenediamine, 2-methoxy-6-chloro-9-(3-(2-chloroethyl) aminopropylamino) acridine *x* 2HCl, 4-nitroquinolone-*N*-oxide, benzo[α]pyrene (B[α]p) and sodium azide). At higher concentrations, some of these agents inhibited the bioluminescence of *V. fischeri*.

This study showed that both mycotoxins effects were delayed and were significant after 10h of incubation. Toxicity test based on the inhibition of *V. fischeri* luminescence would be interesting to investigate for the detection of aflatoxin B_1. For DON, the enhancement of bioluminescence refers to a DNA-damaging effect, and as shown by Czyz *et al.* (2002) mutagenic chemicals were often toxic only at relatively high concentrations. These results confirmed the existence of a relationship between the light emission by *V. fischeri* and the mycotoxin, but further investigations are required to better understand the effects we observed in order to set bioassays for those mycotoxins.

Acknowledgements

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Effects of steam and lactic acid treatments on inactivation of *Listeria innocua* surface-inoculated on chicken skins

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ABSTRACT

Effectiveness of combined steam (10 and 60 s, 70 °C and 98 °C) and chemical treatments, using concentrated solutions of lactic acid (1 and 30 min, 5% and 10% lactic acid), on the inactivation of *Listeria innocua* inoculated on the surface of chicken skins have been studied. Surviving bacteria on the skin were enumerated immediately after treatment, and after 7 days of storage at 4 °C. The most effective treatment was the combination of steam of 98 °C and 10% lactic acid with its immediate efficacy being mainly attributed to the applied heat treatment. However, after 7-day storage, the treatment's effectiveness was mainly due to the applied acid treatment, which prevented growth of the bacteria that survived the heat treatment. Milder treatments (70 °C steam, 5% lactic acid) revealed a genuine synergy between the heat and acid treatments, paving the way for an effective means of reducing bacterial load on the surface of poultry without affecting the product's "raw" appearance.

1. Introduction

Raw poultry carcasses are often heavily contaminated with pathogens belonging to the genera *Listeria* spp., *Salmonella* spp. and *Campylobacter* spp. (Uyttendaele et al., 1998). Among the currently available decontamination treatments, heat and acid treatments have so far shown particular promise (James and James, 1997; Avens et al., 2002).

Since most poultry products are sold raw, mild heat treatments with low steam temperatures of between 71 °C and 75 °C have been applied in order for the quality of the product to be retained (Klose et al., 1971). However, the treatment times used of several minutes were too long to fit in with food processing production rates. The same kind of steam temperature has been applied shortly (18 s) on beef carcasses at industrial scale leading to a 1–2 log reduction of the initial microbial population (Corantin et al., 2005). A European project aimed to analyse kinetically the effect of hot air flows and steam flows on bacteria inactivation (James and Evans, 2006) proved that steam inactivated bacteria more efficiently than hot air. Microbial inactivation kinetics have been evaluated for *Escherichia coli* O157:H7 and for *Salmonella typhimurium* DT104 at the surface of chicken skin subjected to a steam flow of 100 °C (McCann et al., 2006), and have shown an exponential reduction of the microbial population with a rapid 3 to 4-$\log_{10}$ destruction within the first 20 to 30 s of application of the treatment. After the European project, inactivation of *Campylobacter jejuni* AR6 and *E. coli* K12 on the surface of poultry carcasses by steam at atmospheric pressure and hot water treatments have been analysed in relation to product damaging at three different treatment times: 10, 12 and 20 s respectively (James et al., 2007). Maximum decontamination (about 3 $\log_{10}$ cfu cm^{-2}) without skin damaging was found by combining steam, hot water and a rapid cooling of product surface. Jets of steam, superheated or non-superheated, have also been applied for a period up to 60 s to decontaminate disks of poultry skin, surface-inoculated with *Listeria innocua* (Kondjoyan and Portanguen, 2008b). Superheated steam was clearly more effective against the pathogen than non-superheated steam, leading to an average reduction of more than 5 $\log_{10}$ cfu cm^{-2} after 30 s of treatment. The use of superheated steam may prove useful in industrial settings for treatment durations of 10 s to 30 s. This procedure can be a highly effective surface decontamination intervention that does not result in cooking the meat, and a process patent has thus been filed (No. 05 53451).

Another method of decontaminating the surface of food products involves organic acids solutions. Lactic acid is, at first sight, a good candidate as it is authorised for food products, is inexpensive, and in dilute solution does not have an unpleasant odour or taste. A 5-min treatment with a 1% solution of lactic acid gives an immediate reduction of the order of 0.5 to 1 $\log_{10}$ for *Listeria monocytogenes* inoculated on chicken skin (Gonzalez-Fandos and Dominguez, 2006). The reduction is

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of the order of 1 $\log_{10}$ when the concentration of lactic acid is increased to 2% or 4%. Increasing the time to 15 min and the solution temperature to 55 °C leads to a reduction of 2.38 $\log_{10}$ on chicken breast meat (Goncalves et al., 2005). There are hardly any results on solutions with concentrations of lactic acid above 5%, and no systematic study has been conducted to evaluate the effect of contact time on decontamination.

An approach combining heat treatment and organic acid treatments has been used in the case of vacuum-packed, dried or marinated meat (Koutsoumanis and Sofos, 2004; Ikeda et al., 2003; Calicioglu et al., 2002, 2003) or frankfurters (Murphy et al., 2005). Heat treatments give immediate and significant reductions in bacterial counts, while acid treatments lead to bactericidal or bacteriostatic effects that are maintained during storage. Thus an approach combining heat treatment and organic acid treatments seems also particularly promising for carcasses and fresh products which have not been vacuum-packed, marinated, or dried.

This study aimed at identifying the best way of combining steam and lactic acid treatments in order to obtain a method for decontaminating raw poultry carcasses that would be suitable for use at food processing plants, and that would present maximum efficiency without adversely affecting the sensory qualities of the raw product (taste, appearance, texture, colour, and odour). Such a decontamination method should achieve an immediate reduction of the initial bacterial load, and also provide an effective protection of food products during cold storage. Studies have shown that *Listeria* spp. strains are more resistant to decontamination treatments than most other pathogens, in particular *Salmonella* spp. and *Campylobacter* spp. (Hwang and Beuchat, 1995; Sörqvist, 2003; Fernandez et al., 2007). Since *L. monocytogenes* is a major human pathogen it was not reasonable to use this bacterium in an experimental procedure which was expected to be transferred at a pilot scale in a processing plant. Thus instead of *L. monocytogenes*, *L. innocua* was used in this study; a non-pathogenic species, which has been proved to be very close physiologically to *L. monocytogenes* (Begot et al., 1997; Vaz-velho et al., 2001) and has been used as a model of the pathogen in several other tested processes (Antwi et al., 2007). The strains chosen in this study *L. innocua* CLIP 20595 and *L. monocytogenes* 14 were isolated from a meat processing plant and thus found appropriate to study decontamination treatments which have to be applied on a meat and in abattoirs. Preliminary experiments were done to compare the thermal resistance of *L. innocua* CLIP 20595 to that of *L. monocytogenes* 14.

2. Materials and methods

2.1. Inoculation

The strains of *L. innocua* CLIP 20595 and *L. monocytogenes* 14 were stored on microspheres at −18 °C. It was then transferred to an inclined TSA agar tube (Difco, USA) and incubated for 8 h at 37 °C. Two inoculating loops were then transferred to 100 ml of meat medium (meat peptone 10 g/l from Merck, Germany; yeast extract 5 g/l from Difco, USA; Glucose 5 g/l from Prolabo). This culture was incubated for 20 h at 20 °C, and then used to inoculate the chicken skin samples or to compare thermal resistance in liquid medium and on Teflon® slide. Preliminary (unpublished) experiments showed that the cells were at the stationary growth phase.

The skins used were taken from chicken upper legs purchased from a supermarket. The chickens were slaughtered 1 to 3 days before the experiments were performed. The skin samples were excised under sterile conditions and placed on a Teflon® spacer. A Teflon® mesh was deposited in a Petri dish filled with just enough bacterial suspension to cover its top. The spacer was placed on the mesh in such a way that 20 cm^2 of the outer skin surface was in contact with the bacterial suspension for 1 min, without agitation. The skin was then drained for 10 min. During the inoculation, skin and bacterial suspension were kept at room temperature (20 °C).

2.2. Comparison of the thermal resistances of the Listeria

Thermal inactivation of *L. innocua* CLIP 20595 was compared to that of *L. monocytogenes* 14 at the same temperature (either 60 °C or 70 °C) both in a liquid medium and a solid surface.

2.2.1. Thermal inactivation of free-floating (in liquid suspension) bacteria

To determine accurately the heat resistance of the strain of *L. innocua* CLIP 20595 and *L. monocytogenes* 14 suspended in a Tryptone Salt liquid medium (pH = 7.0), 100 µl of the cell suspension (7 $\log_{10}$ cfu ml^{-1}) was sealed in sterile glass capillary tubes (total length: 125 mm, external diameter: 2 mm, internal diameter: 1.6 mm; ringcaps® Hirschmann Laborgeräte, Fisher Bioblock Scientific, Illkirch, France). Capillary tubes were used to obtain a rapid and uniform heat transfer through the column of cell suspension. The tubes were immersed in a thermostat controlled circulating water bath (Lauda 30 l type B T25015, Konigshofen, Germany) at 60 °C. This temperature was chosen for logistic reasons, so inactivation proceeded not too fast nor too slow. At regular times (between 0 and 300 s), one capillary was removed from the water bath and plunged immediately into an ice-water bath to stop the heat treatment. Come-up times, which were in any case very small, were included as part of the total heating time used to calculate the inactivation parameters. Decimal serial dilutions of the samples were made in TS medium and surface plated in duplicate on a selective medium, PALCAM agar (Merck). The use of a selective medium as PALCAM has the drawback to prevent some of the heat-injured bacteria to grow, leading to an overestimation of the decontamination effect. But it was preferred to a non-selective medium (Plate Count Agar or Tryptone Soy Agar) which could have bias the results due to the further contamination of the treated sample by the air flows coming from the cooling system and the room which were not sterile. One hundred microliters of the solution was rolled on the agar plates by using glass spheres (diameter of 3–4 mm). Plates were incubated for 24–48 h at 37 °C and colony-forming units (CFU) were enumerated. The microbial detection limit of the methods was 2.47 $\log_{10}$ cfu ml^{-1}. This limit was set considering that the numerations leading to less than 30 CFU on one Petri dish were not enough reliable.

2.2.2. Thermal inactivation on surface

A nonbiological and well-known surface, Polytetrafluoroethylene or Teflon® has been chosen in a first stage to compare the heat resistance of *L. innocua* CLIP 20595 and *L. monocytogenes* 14 attached to solid surfaces. This material has a smooth and hydrophobic surface and thermal characteristics close to those of poultry skin. The Teflon® sheets were cut into rectangular pieces (1 × 3 cm, 1-mm thick). Before each experiment, the Teflon® slides were soaked for 10 min in a 2% solution of the commercial surfactant TFD4 (Francklab S.A. Saint-Quentin en Yveline, France) and rinsed five times for 5 min with hot tap water and five times for 5 min with demineralised water. Finally, the surfaces were autoclaved for 15 min at 121 °C. To contaminate Teflon® slides, *Listeria* spp. cells were attached to the surface by sedimentation at 20 °C for 2 h. Twenty-five milliliters of the bacteria suspension was poured into a Petri dish containing four Teflon® slides (1 control + 3 tests). After the contact period, nonadherent cells were eliminated by five consecutive rinses with 25 ml of sterile tryptone-salt medium. Trials were performed on poultry skin including several rinses and no rinse of the control and of the sample before the treatments. Different contact times between inoculum and control/sample were also tested to favour adhesion (between 5 min and 2 h). In our case these conditions modified slightly the initial bacteria concentration on the control and on the sample but did not affect the result of the heat/acid treatment. In the following the inoculation time was set at 10 min and inoculation was not followed by any rinsing stage. After the thermal treatment each decontaminated Teflon® slide and the control (untreated) was introduced into tubes containing TS medium. The detachment of the surviving cells was performed by

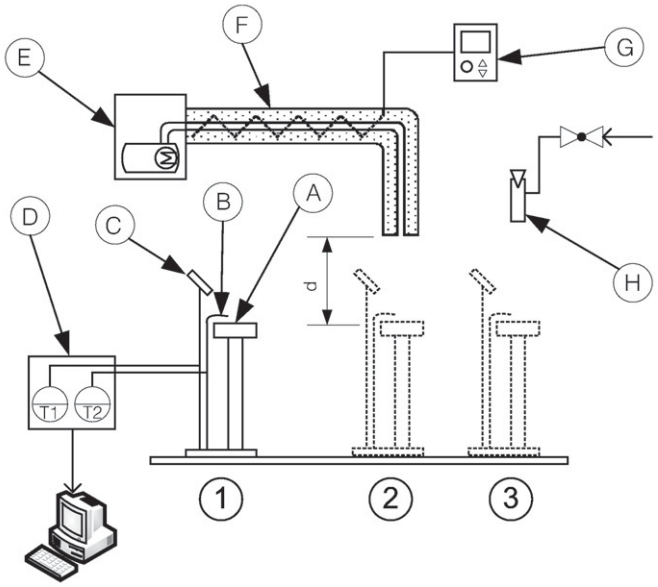

Fig. 1. Schematic representation of the functioning of the superheated steam rig. A) Sample holder; B) Thermocouple; C) Infra-red pyrometer; D) Data acquiring system; E) Steam generator; F) Pipe with heating resistances; G) resistances regulation; H) Cooling system; 1) Sample holder positioning; 2) Steam treatment; 3) Cooling.

sonication (2 min—Deltasonic, Meaux, France). Preliminary experiments on poultry skin had shown that the results were the same using a stomacher (no additional lethal effect due to sonication). After serial dilution in TS medium, the number of adherent and viable cells was enumerated on PALCAM agar (incubation for 24–48 h at 37 °C). The solution was spread on the dishes using the spiral technique and the microbial detection limit was 2.7 $\log_{10}$ cm^{-2}.

2.2.3. Calculation of D-value

D-values (decimal reduction time, or time required to inactivate 90% of the population) were calculated as the inverse slope of the linear portion of survivor curves (obtained by plotting decimal logarithms of survival counts versus their corresponding heating times). Linear regression lines were fitted to the linear portion of two sets of independent data.

2.3. Heat treatment

A detailed description of the heat treatment apparatus was given in Kondjoyan and Portanguen (2008a). Steam produced from a generator at 150 °C–165 °C passed through a stainless steel pipe surrounded by two heating resistors and insulated with rock wool. Controlled heating of the pipe was produced by electrical resistances thus generating steam with no condensates. The steam jet was released outside the pipe thus mixing with air before impinging on the surface of sample (Fig. 1).

When the sample was poultry skin, it was located on a cylindrical support made of Teflon®. During preliminary experiments with Teflon® slides, the slide was fixed on the sample holder with double face Scotch® tape. Before experiments surface of sample was made flush to that of the support. Distance *d*, between the surface of the sample and the outlet of the pipe was fixed accurately using a manual traversing system. The temperature at the surface of the sample was measured by and Infra-Red thermometer in a spot of 2 cm in diameter located at the centre of sample. The measuring part of the Infra-Red system was attached to the sliding device to be able to measure the surface temperature of sample all along the decontamination treatment. Temperature of the impinging jet was measured every second using a 0.6 mm thick thermocouple (of type K) located 3.0 mm above the middle of sample surface. Thermocouple and IR thermometer were calibrated using procedures already described elsewhere (Kondjoyan and Portanguen, 2008a).

At the end of the heat treatment the surface of the sample was rapidly cooled by sliding the sample under a 45–55 m s^{-1} jet flow of cold air (temperature 3 °C–5 °C), produced by a Ranque-Hilsch tube ("vortex cooler"). The three positions of the sliding system: sample away from the steam jet, subjected to jet and subjected to cold air were perfectly fixed using blocking ball bearings. Apparatus was kept under the same heating conditions for at least 2 h to reach steady-state conditions before experiments began.

Steam condensed on the surface of the colder sample during the first seconds of the treatment. When the temperature of the steam jet reaching the sample was greater than 98 °C (boiling point at an elevation of 800 m) the condensate film evaporated and the surface temperature remained constant and equal to the boiling temperature. This physical phenomenon ensured that the temperature stayed steady at 98 °C throughout the experiment (Fig. 2). Increasing the distance between the steam outlet nozzle and the sample cooled the jet by introducing more air thus giving a steam jet temperature of less than 98 °C (in our case, 60 °C to 70 °C at the surface of the sample).

2.4. Acid treatment

A 44.6-mm diameter skin disk was placed between a Teflon® support and a stainless steel dish. The assembly was secured by clamps. Two holes drilled at the top of the dish allowed filling and draining of the dish. The skin was placed in contact with the acid solution (lactic acid with a concentration of 5% v/v or 10% v/v, VWR International, France) for 1 min or 30 min at room temperature (20 °C). Following treatment, the decontamination solution was eliminated. No rinsing was performed.

2.5. Enumeration

The surviving bacteria on the skin were enumerated either immediately after treatment (Day0), or after the skin had been stored for 7 days at 4 °C in Petri dishes closed with self-sealing plastic film (Day7). Inoculated, but untreated, controls were made at Day0 and Day7. Preliminary experiments had shown that putting inoculated skins in contact with sterile physiological water for 30 min with the same setup as used during acid treatment has no significant effect on its bacterial concentrations. Untreated controls were not rinsed before enumeration or storage.

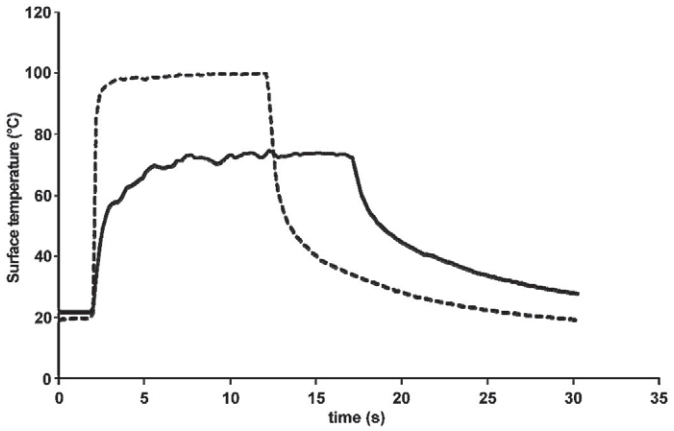

Fig. 2. Temperature of the skin surface, for two significant trials. Solid line: 15-s treatment at 75 °C. Dotted line: 10-s treatment at 98 °C. Product is placed under steam jet 2 s after starting measurement.

To enumerate the surviving bacteria, a 35-mm diameter disk was cut out of the skin and homogenised for 1 min in 35 ml of buffered peptone water (Biokar Diagnostics, France). Tenfold dilutions were made in sterile saline (0.85% NaCl) and spiral plated on PALCAM agar (Merck KGaA, Germany) in duplicate. In order to lower the detection threshold as far as possible, 1 ml of the homogenate was pour plated in the melted PALCAM agar. The temperature of the melted agar which was initially 50 °C decreased rapidly below 45 °C (maximum growth temperature of *L. innocua*) when poured in the Petri dish. Thus it was reasonable to think that this second heat exposure should not affect significantly the survival of bacteria. However some experiments were performed using also surface-spreading cultures to check this assumption. No significant differences were found between the results coming from the two types of cultures. Thus melted agar pouring was kept to be able to calculate the important decontamination effects presented in this paper. For each sample, the concentration of *L. innocua* was expressed in cfu cm^{-2}, and the $\log_{10}$ of this value was used to calculate the decimal reduction of the bacterial population. The detection threshold was 1.0 $\log_{10}$ cfu cm^{-2}.

2.6. Experimental design

The inoculated skins were decontaminated with steam used alone, with lactic acid used alone and by a successive application of these two treatments. The surviving bacteria were enumerated either immediately after treatment, or after 7 days of storage at 4 °C. All the experiments were repeated 10 times in order to reduce the heterogeneity effect of the raw material, thereby reliably quantifying the efficacy of the different decontamination conditions.

The selected order of treatment application was heat treatment followed by acid treatment. Applying an acid treatment before the heat treatment would have the disadvantage of leaving a liquid film on the surface of the sample which could provide resistance to the subsequent heat treatment. From a food processing standpoint, applying an acid solution after the heat treatment would rapidly cool the surface of the product thus eliminating any risk of cooking the skin or underlying muscle. Furthermore, O'Driscoll et al. (1996) showed that an acid stress increases the resistance of *L. monocytogenes* to subsequent thermal shocks (54 °C in liquid media). This is comforted by the results of Koutsoumanis and Sofos (2004) who observed that during a hot water (75 °C) treatment followed by an acid lactic treatment (2% at 55 °C) the heat–acid sequence is generally more efficient that the acid–heat sequence. To the best of our knowledge, the inverse phenomenon has never been observed with *Listeria* spp.

Three separate heat treatments (designated as groups A, B and C) were applied in order to evaluate the impact of time and temperature on the efficacy of the procedure. These conditions were selected based on actual conditions at food processing plants (required production rates) and the results were given in the literature.

Biphasic bacterial inactivation has been observed by McCann et al. (2006). During steam pasteurisation, the number of bacteria fell rapidly during the first 10 s to 30 s of steam treatment, followed by a period of "tailing". Two types of short heat treatment, with potential benefits in terms of food processing production rates, were studied: 10 s at 98 °C (group A) and 15 s at 70 °C (group C). A longer treatment time, which ensured that the tailing phase was reached, was studied to estimate the effect of low temperature heat treatments (treatment B).

For acid treatments, an initial high concentration of lactic acid (10%; application times 1 min and 30 min) was selected to effectively discriminate between the effect of the lactic acid used alone compared with that of the combined treatments. A second concentration level (5%, 1 min) was selected to obtain milder treatments which would reveal any possible synergies between the heat and acid treatments.

Statistical analysis was carried out using Statgraphics Centurion XV software (StatPoint Inc.). To discriminate between the results of the different treatments, the least significant differences (LSD) procedure of Fischer was used with a confidence threshold of 95%. The estimated repeatability for the $\log_{10}$ reduction was the deviation observed for the ten measurements of average bacterial concentration.

3. Results and discussion

3.1. Comparison L. innocua to monocytogenes

During the preliminary experiments performed in a liquid medium *L. innocua* CLIP 20595 was found to be more heat resistant than *L. monocytogenes* 14 with $D_{60\,°C}$ values of 49 s and 28 s respectively. This result was confirmed on Teflon® slides leading to average reductions of *L. innocua* and *L. monocytogenes* numbers at 70 °C of 2.7 $\log_{10}$ cfu cm^{-2} and 1.5 $\log_{10}$ cfu cm^{-2} respectively. Thus it was considered that the reduction of the population of *L. innocua* CLIP 20595 will, at least, lead to the same reduction of the population of *L. monocytogenes* 14.

Fig. 2 illustrates the skin surface temperature for treatments of 10 s at 98 °C, and 15 s at 70 °C. The phases of rising temperature, constant temperature and cooling can be clearly distinguished. For treatments at 98 °C, the time taken for the temperature to rise was less than 0.4 s; the surface temperature then remained constant throughout the duration of the treatment. The time taken for the temperature to rise was longer for treatments at 70 °C, of the order of 3 to 7 s. In addition, surface temperature fluctuations of several degrees were recorded due to the increased distance between the steam outlet nozzle and the sample, which generates both turbulence in the jet flow and greater sensitivity of the jet direction to air movements in the room. These jet flow fluctuations do not result in a standard deviation increase on measurements of bacterial death (Table 1).

Immediately after inoculation, the concentration of *L. innocua* on the surface of the controls was 6.65 ± 0.16 $\log_{10}$ cfu cm^{-2}. After 7 days of storage at 4 °C, this concentration had risen to 7.11 ± 0.33 $\log_{10}$ cfu cm^{-2}. Regarding experimental uncertainty, there was no significant difference between the enumerations at Day0 and the enumerations at Day7 (Table 1).

3.2. Group A

After a 10-s heat treatment at 98 °C, the reduction in bacterial count reached 4.32 $\log_{10}$ cfu cm^{-2} (Table 1). The bacterial concentration was below the detection threshold in 4 cases out of 10. This reduction in bacterial count was greater than that reported in the literature for other bacteria and for slightly lower treatment temperatures (i.e. 85 °C–90 °C). McCann et al. (2006) obtained decimal reductions of 2.91 $\log_{10}$ cfu cm^{-2} of *E. coli* O157:H7 and *S. typhimurium* DT104 after treating chicken skin samples with steam at 87 °C for 10 s. Whyte et al. (2003) observed reductions of 0.61 $\log_{10}$ cfu g^{-1} of enterobacteria found on raw chicken carcasses after being steam-treated for 12 s at 90 °C. James et al. (2000) observed a reduction in bacterial count of 1.22 $\log_{10}$ for skin-on chicken breast samples inoculated with *E. coli* O80 and steam-treated for 10 s (surface temperature of 98 °C).

We think that the powerful steam jet used in this study could have generated more intense transfers on the surface of the product compared to those in the experiments by McCann et al. (2006) and Whyte et al. (2003).

Regarding the application of 10% lactic acid for 1 min or 30 min, the immediate post-treatment reduction in bacterial counts was significantly lower than that observed with heat treatment used alone (Table 1). Nevertheless bacterial reductions remained greater than 1.0 $\log_{10}$ cfu cm^{-2}, reaching 1.47 and 2.45 $\log_{10}$ cfu cm^{-2} for treatments of 1 min and 30 min respectively. To the best of our knowledge, the lactic acid concentration used in this study is greater than that used so far in the literature. This study obtained a greater population reduction than that observed by Gonzalez-Fandos and Dominguez (2006) who, for treatments lasting 5 min with 5% lactic acid, obtained reductions of the order of 1.05 $\log_{10}$ g^{-1}. The 2.45 $\log_{10}$ cfu cm^{-2} reduction is

Table 1
Measured bacterial concentrations and log reductions

Treatment	Mean bacterial concentration on the skin (log cfu cm^{-2}) with confidence interval 95%		Mean log reduction (log cfu cm^{-2})		Number of trials under detection (10 repetitions)	
	Day0	Day7	Day0	Day7	Day0	Day7
Control	$6.65 \pm 0.16^{a,b}$	7.11 ± 0.33^{b}	–	–	–	–
Group A						
Heat 98 °C 10 s	$2.33 \pm 0.78^{c,d}$	$3.53 \pm 0.97^{e,f}$	4.32	3.57	4	2
Acid 10% 30 min	$4.20 \pm 0.57^{f,g}$	$<1.00^{h}$	2.45	>6.11	0	10
Acid 10% 1 min	$5.18 \pm 0.23^{g,i,j}$	$1.85 \pm 0.83^{c,d,h,k}$	1.47	5.25	0	6
Combined: Heat 98 °C 10 s+Acid 10% 30 min	$2.09 \pm 0.8^{c,d,k}$	$<1.00^{h}$	4.55	>6.11	5	10
Combined: Heat 98 °C 10 s+Acid 10% 1 min	–	$1.40 \pm 0.73^{c,h,k}$	–	5.71	–	8
Group B						
Heat 70 °C 1 min	$1.20 \pm 0.32^{h,k}$	$1.8 \pm 0.56^{c,d,h,k}$	5.44	5.30	8	4
Combined: Heat 70 °C 1 min+Acid 10% 30 min	$1.13 \pm 0.25^{h,k}$	$<1.00^{h}$	5.52	>6.11	9	10
Combined: Heat 70 °C 1 min+Acid 10% 1 min	–	$<1.00^{h}$	–	>6.11	–	10
Group C						
Heat 70 °C 15 s	$5.16 \pm 0.42^{g,i,j}$	$5.38 \pm 1.08^{i,j}$	1.49	1.72	0	1
Acid 5% 1 min	$5.93 \pm 0.14^{a,j}$	$4.6 \pm 0.72^{g,i}$	0.71	2.54	0	0
Combined: 70 °C 15 s+5% 1 min	$5.71 \pm 0.62^{a,j}$	$2.80 \pm 0.87^{d,e}$	0.93	4.30	0	2

Mean bacterial concentrations with same superscript (^{a,b}) are not significantly different.

comparable to that obtained by Goncalves et al. (2005) on chicken breast meat subjected to 4% lactic acid solution at 55 °C during 15 min (2.38 $\log_{10}$ cfu cm^{-2}).

For combined treatments (10-s heat treatment at 98 °C followed by 10% lactic acid treatment lasting 30 min), the average decimal reduction observed at Day0 did not significantly differ from the decimal reduction observed for heat treatment used separately (Table 1). In view of the similarity between heat treatment used alone and its combination with 10% lactic acid for 30 min, it was decided not to continue with combined experiments at Day0 for shorter contact time (1 min).

After 7 days of storage at 4 °C, we observed higher bacterial growth after a 10-s heat treatment at 98 °C than on the control. The number of samples with bacterial concentrations below the detection threshold decreased during storage. However, regarding our study, the efficacy of the applied heat treatment was such, that the observed subsequent growth of the surviving bacteria does not negate the potential benefits of this treatment on the contrary to what has been observed in other studies (James et al., 2000). There is some doubt though as to whether this treatment would be of equal value in a slaughterhouse environment, where greater incidence of airborne contamination is likely. Mechanisms involving the Heat Shock Proteins (HSP) proteins synthesis and the cellular recovery (Pagán et al., 1997; Abee and Wouters, 1999; Gahan et al., 2001; Miller et al., 2006) or the decrease in competitive microflora may have encouraged the growth of surviving bacteria during storage at 4 °C knowing that *Listeria* species have psychrotrophic nature. On the other hand, the Jameson effect, which refers to the suppression of growth of all microorganisms by high total microbial loads (Gimenez and Dalgaard, 2004), may be associated with the fact that no significant growth was observed in untreated samples after 7 days of storage.

For acid treatments used alone, we observed a marked bactericidal effect during storage; this effect was not observed when heat treatment was used alone. In particular, for treatment durations of 30 min, the number of surviving *L. innocua* was systematically below the detection threshold, in each of the 10 repeat experiments. In this case, the reduction in bacterial count was recorded as greater than 6.11 $\log_{10}$ cfu cm^{-2}. In the case of 10% lactic acid treatment for 1 min, bacterial death was not total and the number of surviving *L. innocua* only fell below the detection threshold in 6 cases out of 10. The reduction in bacterial count nevertheless reached a value of 5.25 $\log_{10}$ cfu cm^{-2} on average. It is probable that the residual lactic acid content makes it possible for the decontamination process to continue during storage. Gonzalez-Fandos and Dominguez (2006), who applied treatments for 5 min with 5% lactic acid on *L. monocytogenes* inoculated onto the surface of chicken legs, found lower reductions, of the order of 1 $\log_{10}$ at Day0 and 1.74 $\log_{10}$ at Day7.

The average decimal reductions observed 7 days after application of the combined treatment did not differ significantly from those observed after acid treatment used alone (Table 1). On the other hand, they were significantly greater than those observed for heat treatment used alone. This is due to continued chemical decontamination during refrigerated storage. Koutsoumanis and Sofos (2004) demonstrated that resistance to acid shock is temperature dependent, and also showed that in liquid media, *L. monocytogenes* was less resistant to acid treatments applied at 4 °C than to those applied at 30 °C. For the combined procedure, the overall death effect at Day7 can therefore mainly be attributed to the acid treatment. However, the number of uncountable samples made it difficult to identify possible synergy effects between heat and acid treatments.

In the situations described above, the immediate effect (Day0) of combined treatments can be mainly attributed to heat treatment at 98 °C, which gives greater decontamination efficacy than the acid treatment. The acid treatment, however, exhibits a longer effect that extended bacterial decontamination during the 7-day storage period at 4 °C. The combined treatment therefore benefits from both types of treatment as its effect was always, greater than, or equal to, that of each treatment applied separately.

3.3. Group B

Immediately after heat treatment at 70 °C for 1 min, the reduction in the number of *L. innocua* present on the skin reached 5.44 $\log_{10}$ cfu cm^{-2}, the number of *L. innocua* was below the detection threshold in 8 cases out of 10 (Table 1). Therefore this treatment proved to be significantly more effective than the heat treatment applied to group A (10 s only at 98 °C) (Table 1). This result can be explained by the fact that after 10 s the treatment at 98 °C was still a long way from having reached its maximum efficacy level (first rapid population reduction phase) while a treatment of 60 s at 70 °C reached the tailing phase, during which the only survivors were the microbial population that were resistant to the applied heat treatment, and which were found in only limited numbers on the chicken skin (McCann et al., 2006). The heat inactivations obtained in this study at 70 °C were greater than

those recorded by Klose et al. (1971) for similar temperatures but with longer treatment durations (4 min). As the reduction values presented by Klose et al. (1971) were obtained using a very different microbiological and heat treatment protocol with a low number of repetitions, it is difficult to discuss this deviation further. The measurements after 7 days of cold storage showed a very slight increase in the average bacterial population. Although, at an order of 0.6 $\log_{10}$ (Table 1), this growth was statistically insignificant, the number of cases with enumerations below the detection threshold was reduced by half (4 cases out of 10 after 7 days of storage against 8 cases out of 10 immediately after treatment). This would appear to reveal a slight regrowth of the population during the storage phase after heat treatment for 1 min at 70 °C, which could, perhaps, be demonstrated with a lower detection threshold.

When treatment at 70 °C was combined with the strongest acid treatment (i.e. 10% lactic acid for 30 min), decimal reductions immediately after treatment were not significantly different from those observed for heat treatment alone (Table 1). After 7 days of storage at 4 °C, the remaining bactericidal effect related to the acid treatment (1 and 30 min) resulted in greater bacterial destruction than that observed for heat treatment used alone. Decreasing the contact time from 30 min to 1 min gave the same reductions in bacterial count although the combined treatment tended to increase the number of uncountable samples (10 cases out of 10, Table 1) compared to acid treatment used alone (only 6 cases out of 10 for 1 min, Table 1). The limits of the enumeration techniques make it impossible to tell whether, at this level, there is an actual synergy between the 70 °C heat treatment and the 10% lactic acid treatment.

A 1-min heat treatment at 70 °C decontaminates the samples without significantly altering the skin texture.

In order to reveal possible synergies between the heat and acid treatments, we combined treatments that, when used separately, would have a lesser efficacy than those used in earlier experiments. The duration of the 70 °C heat treatment was reduced from 1 min to 15 s. Given the duration of the initial phase of temperature rise, this time corresponded to an effective treatment time of 10 s at 70 °C (Fig. 1). Furthermore, the lactic acid concentration was brought back to 5%, while maintaining a treatment time of 1 min.

3.4. Group C

The variability in concentrations of *L. innocua* on the surface of chicken skins after short-duration heat treatment at 70 °C was of the same order of magnitude as that observed at 98 °C, whereas we would have expected it to be greater due to the increase in temperature fluctuations. In addition, enumerations of bacterial counts after 15-s treatments at 70 °C should theoretically have been more accurate as there were a greater number of samples with a *L. innocua* concentration above the enumeration threshold than before.

At 70 °C, the decimal reduction immediately after heat treatment (1.49 $\log_{10}$ cfu cm^{-2}, Table 1) was significantly lower than that obtained at 98 °C for similar 10-s treatment duration (4.32 $\log_{10}$ cfu cm^{-2}, Table 1). The result obtained at 70 °C was close to that of 2.11 $\log_{10}$ measured by Logue et al. (2004) under similar conditions (10 s at 75 °C) but using different substrate and bacteria (inoculated beef muscle, *E. coli* O157: H7). There was almost no bacterial growth during the storage phase in contrast to what has been said in literature for the 30 s-pasteurisation of beef carcasses at 75 °C (Corantin et al., 2005) and what was observed previously for the 10-s treatment at 98 °C (Table 1). As the 70 °C treatment was much gentler than the 98 °C treatment, the bacteria concentrations recorded at Day0 were close to the initial concentrations recorded on the untreated control (5.16 $\log_{10}$ and 6.65 $\log_{10}$ cfu cm^{-2} respectively). Therefore, based on the "Jameson effect" mentioned previously, this absence of growth during the 7-day storage could be mainly attributed to the existing high bacterial load resulting in bacterial competition phenomena. Moreover a possible regrowth would have been limited by the storage temperature of 4 °C. Ikeda et al. (2003) observed no growth of *Listeria monocytogenes* during the vacuum storage at 4 °C of beef slices previously treated with hot water at 75 °C while an important regrowth occurred for storage temperature of 10 °C.

During acid treatment used alone, reducing the acid concentration from 10% to 5% halved the immediate efficacy of the treatment (1.47 and 0.71 $\log_{10}$ cfu cm^{-2} respectively). This difference is not significant, however, given the low level of the effects observed and the standard measurement deviation (Table 1). Bacterial inactivation continued following treatment, reaching 2.5 $\log_{10}$ cfu cm^{-2} after 7 days of storage. This bactericidal effect was not observed by Gonzalez-Fandos and Dominguez (2006) who only identified a slight inhibition effect.

When a 70 °C, 15-s heat treatment was combined with 5% lactic acid treatment for 1 min, the immediate post-treatment reduction in the number of *L. innocua* was not significantly different from that observed after heat treatment used alone or acid treatment used alone. This demonstrates that there is neither any synergistic, nor even any additive effect between the two treatment types at Day0. Conversely, after 7 days of storage, the decimal reduction obtained using the combined treatment was approximately twice as high as that of each treatment applied separately (4.30 as opposed to 1.72 and 2.54 $\log_{10}$ cfu cm^{-2} respectively). In this case, there was a genuine synergy effect between the heat and acid inactivation processes which is particularly promising from a food processing standpoint. The heat treatment did not alter the skin texture, while the combined effects reduced the *L. innocua* concentration at the surface of the raw carcasses by over 4.0 $\log_{10}$ cfu cm^{-2} after 7 days of storage. These two treatments, which would be unsatisfactory, used separately, lead to a significant reduction of the initial microbial population when they are combined. This combination which could be easily implemented in food processing plants presents the advantage to be gentle on food products and to use low quantities of chemical additive (less residues and operational costs).

4. Conclusion

The results obtained demonstrated that, as would be expected, the efficacy of heat treatments increased with surface temperature or treatment duration. The most marked reductions in bacterial count were obtained by applying a 1-min treatment at 70 °C; shorter treatments at higher temperatures also gave significant reductions. However, heat treatment used alone did not prevent risks of later bacterial growth during storage, as observed After 7 days of storage at 4 °C for the most effective heat treatment (98 °C, 10 s). Lactic acid treatments did not give such large reductions in bacterial count at Day0 as heat treatments. However they all have presented a remaining bactericidal effect during the storage period at 4 °C.

Combined decontamination treatments can cumulate the respective advantages of heat and acid treatments as their effect is always greater than, or equal to, that of each treatment applied separately. After 7 days of storage at 4 °C, no significant synergy was clearly observed for the 10% lactic acid treatments. On the other hand, this effect was clearly identified when milder heat and acid treatments were used. The 4 $\log_{10}$ cfu cm^{-2} reduction thus obtained could be decisive in improving the microbial safety and shelf life of poultry products. Such treatments are fast enough to comply with the production rates encountered in industrial slaughter lines. Moreover sensory analyses (not shown) performed on acid treatments lead to conclude that the combined treatments should not affect the product's organoleptic properties.

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Antibiotic resistance in Gram-negative bacteria isolated from farmed catfish

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Abstract

Feeding practices for *Pangasius sp*. aquaculture in Mekong Delta (Viet Nam) are assessed and the importance of home-made feeding is highlighted. Farmers spend 5% production cost for disease prevention, mainly antibiotics for prophylactic and therapeutic treatments. Therefore, the study aims to analyse the resistance of fish bacteria to antibiotics to help them improve their practices.

Bacteria isolated from catfish ($n = 92$) were arbitrarily-selected from 3 different fish farms to analyse their antibiotic resistance and evaluate the antibiotic pressure exerted on the surrounding environment. Antimicrobial susceptibility was examined for selected isolates against 6 major antibiotics using the agar diffusion method: oxytetracycline, chloramphenicol, trimethoprim-sulphamethoxazole, nitrofurantoin, nalidixic acid, and ampicillin. The predominant bacterial microflora consisted of members of the *Enterobacteriaceae* (49.1%), *Pseudomonads* (35.2%) and *Vibrionaceae* (15.7%) families. The main multiple antibiotic resistance profiles included AM-OTC-SXT-NA (17.8% of isolates), OTC-SXT-NA (15.1%), AM-C-FT-SXT-NA (13.7%), AM-FT-OTC (9.6%), AM-C-FT-OTC-SXT-NA (8.2%). MAR index values of the 3 farms ranged from 0.36 to 0.62 which indicates a high-risk exposed-antibiotic source.

These results showed that antibiotic resistance among fish indigenous bacteria is of a high concern in catfish aquaculture in the Mekong River Delta.

Keywords: *Pangasius* catfish; Feeding; Antibiotic resistance

1. Introduction

In 2002, Asia accounted for 91.2% of the aquaculture production quantity, which averaged 35 496 000 tons (SOFIA, 2004). The impact of the intensive use of antimicrobial agents worldwide for prophylactic and therapeutic purposes has been associated with the increase of bacterial resistance in the exposed microbial environment. Currently, multiple antibiotic resistance has been reported in a wide range of human pathogenic or opportunistic bacteria such as *Campylobacter sp*. (Randall et al., 2003), *Klebsiella pneumoniae* (Carneiro, Silva, Merquior, & Queiroz, 2003), *Salmonella sp.* (Randall, Cooles, Osborn, Piddock, & Woodward, 2004), *Pseudomonas aeruginosa* (Ziha-Zarifi, Llanes, Köhler, Pechere, & Plesiat, 1999), and also in fish pathogens (Schmidt, Bruun, Larsen, & Dalsgaard, 2001; Teuber, 2001). Reservoirs of antibiotic resistance can interact between different ecological systems and potential transfer of resistant bacteria or resistant genes from animals to humans may occur through the food chain (Teuber, 2001; van den Bogaard & Stobberingh, 2000; Witte, 2000). In view of this and because of their broad environmental distribution, indigenous fish microflora are likely important reservoirs to be considered.

To ensure a proper use of antibiotics, standards and methods are developed to monitor and control their residual

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concentration in the fish flesh. However, resistance phenomena are not systematically correlated with the presence of the corresponding drugs (Kerry, Coyne, Gilroy, Hiney, & Smith, 1996). So the evaluation of the resistance potential of indigenous fish bacteria is useful to help farmers and veterinarians setting a more efficient and appropriate farm management for chemicals. It is the responsibility of the scientific community as well to ensure that farmers are supported with appropriate advice on farm management and on environmentally safe ways of using chemicals. From this point of view, the purpose of this article was to provide the farmers with an analysis of the resistance of fish bacteria to antibiotics to help them improve their practices. We propose in this study to examine the susceptibility of arbitrarily isolated bacteria from catfish cage farms in Viet Nam (*Pangasius hypophtalmus*) to 6 major antibiotics used in aquaculture (oxytetracycline, chloramphenicol, trimethoprim-sulphamethoxazole, nitrofurantoin, nalidixic acid, ampicillin). This analysis is essential to provide information on the prevalence of resistance and multiple antibiotic resistance in farmed catfish of this region, which is very important in terms of world fish production.

2. Materials and methods

2.1. Selection of bacteria

Fish samples (*P. hypophtalmus*) were collected in sterile plastic bags from 3 different freshwater (0% NaCl) catfish farms (10 healthy fish/farm, total 30 fish) practising the cage culture (farm names are not revealed and called CF1, CF2 and CF3). The mixture (50 g) from the intestines and gills of fish (10) from each farm were mixed with 450 ml of sterile buffered peptone water and crushed with a Stomacher. Bacterial colonies were arbitrarily isolated on Plate Count Agar (Difco) and purified through successive streaking on PCA plates. Isolates (92) were then recovered as follows: 29 from CF1, 40 from CF2 and 23 from CF3.

All the isolates ($n = 92$) were characterised by Gram staining, oxidase and catalase reactions, microscopic observation, cell motility, glucose metabolism, and then identified by API System strips (Biomérieux at Marcy l'Etoile. France) as follows: API 20E for bacilli Gram negative oxidase negative (mainly *Enterobacteriaceae*), API 20NE for bacilli Gram negative oxidase positive (non-*Enterobacteriaceae*). All API strips were prepared according to the instructions of the manufacturer. Further confirmation of Gram-negative bacteria was achieved by use of Pasteur Institute laboratory tests (Le Minor & Richard, 1993; Richard & Kiredjian, 1995).

2.2. Antibiotic susceptibility

Antibiotic susceptibility was determined by the agar diffusion method according to French national guidelines (SFM, 2004). Bacterial suspensions prepared in sterile 0.85% saline matching an optical density of 0.5 McFarland standard corresponding to 10^8 cfu/ml and diluted 1:100 in physiological saline were inoculated by lawn onto Muller-Hinton agar (Difco, Le Pont de Claix, France). Each antibiotic test was run in duplicate on freshly prepared agar plates. After incubation for 24 h at 37 °C, organisms were classified as sensitive, intermediate or resistant according to the inhibition zone diameter (SFM, 2004). The tested antibiotics were: oxytetracycline OTC/30IU, chloramphenicol C/30 μg, trimethoprim-sulphamethoxazole SXT/1.25 μg–23.75 μg, nitrofurantoin FT/300 μg, nalidixic acid NA/30 μg, ampicillin AM/10IU. Antibiotic discs were purchased from Bio-Rad laboratory (Marnes-la-Coquette, France). Reference strain *Escherichia coli* ATCC 25922 (Institut Pasteur CIP 7624) was used as a control organism.

The MAR index (multiple antibiotic resistance) was determined for each farm according to Krumperman (1985) and is defined as $a/(b \cdot c)$ where "a" represents the aggregate antibiotic resistance score of all isolates from a farm, "b" is the number of antibiotics and "c" is the number of isolates from the farm. A MAR index value of less than or equal to 0.2 is considered to indicate from animals in which antibiotics are seldom or never used. A MAR index value greater than 0.2 is considered indicate from a high-risk antibiotic-exposed source.

3. Results

The isolated fish microflora ($n = 92$) consisted mainly of members of the *Enterobacteriaceae* (49.1%), *Pseudomonads* (35.2%) and *Vibrionaceae* (15.7%) families (Table 1). 82.5% of the isolates from CF2 were enterobacteria (compared to 51.7% from CF1 and 13% from CF3).

Isolates (11) of *E. coli* were recovered from the 3 farms of which 10 isolates were resistant to each of trimethoprim-

Table 1
Identification and source of arbitrarily-selected bacterial isolates ($n = 92$)

Bacterial species	Fish farm			Total
	CF1	CF2	CF3	
Chryseomonas luteola	1			1
Escherichia coli	3	8		11
Escherichia hermanii	4	3		7
Edwardsiella tarda		1		1
Enterobacter cloacae	6		1	7
Enterobacter agglomerans		1		1
Enterobacter gergoviae		5	1	6
Enterobacter sp.			1	1
Pragia fontium		1		1
Proteus vulgaris		2		2
Pseudomonas fluotescens			1	1
Pseudomonas cepacia	3		11	14
Pseudomonas pseudomallei	4	1	3	8
Serratia plymuthica		3		3
Vibrio parahaemolyticus		1		1
Vibrio metschnikovii	3	5	5	13
Xanthomonas maltophilia	3			3
Xenorhabdus nematophilus		1		1
Xenorhabdus luminescens	2	8		10
Total	29	40	23	92

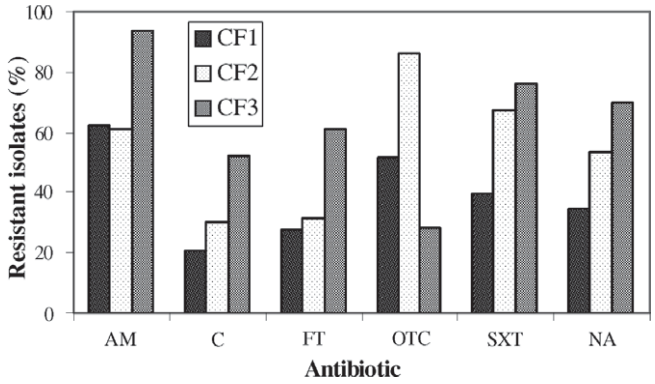

Fig. 1. Percentage of antibacterial resistance for arbitrarily-selected Gram-negative isolates from catfish farms ($n = 92$). CF1: 29 isolates, CF2: 40 isolates, CF3: 23 isolates. Antibiogrammes were carried out for the 92 isolates against 6 various antibiotics: oxytetracycline (OTC/30IU), chloramphenicol (C/30 μg), trimethoprim-sulphamethoxazole (SXT/1.25 μg–23.75 μg), nitrofurantoin (FT/300 μg), nalidixic acid (NA/30 μg), ampicillin (AM/10IU). *n*: number of isolates.

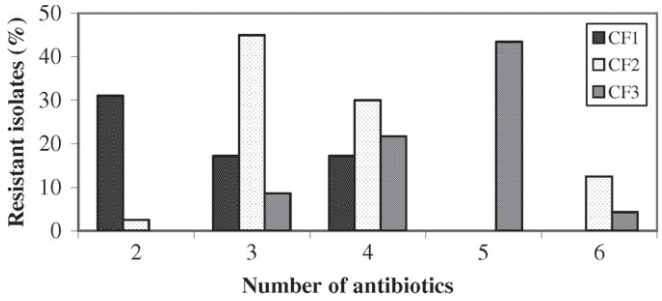

Fig. 2. Multiple antibiotic resistance for arbitrarily-selected Gram-negative isolates from catfish farms (CF1–CF3, $n = 92$). CF: catfish farm, *n*: number of isolates.

sulphamethoxazole and oxytetracycline (90.9%), and 6 isolates were resistant to each of chloramphenicol and ampicillin (54.5%). These isolates ($n = 11$) were all sensitive to nitrofurantoin.

Resistance frequencies were very high and quite similar in the 3 farms: 96.5% of isolates in CF1, 97.5% in CF2 and 95.4% in CF3. Bacterial isolates from CF3 showed the highest resistance rate for ampicillin, chloramphenicol, nitrofurantoin, trimethoprim-sulphamethoxazole and nalidixic acid and the lowest rate for oxytetracycline (Fig. 1). Isolates from CF2 were the most resistant to oxytetracycline with a rate of 86.3%. Bacteria from CF1 had the lowest level of resistance for chloramphenicol, nitrofurantoin, trimethoprim-sulphamethoxazole and nalidixic acid. The average rates of resistance of the 3 farms were 69.6% ampicillin, 60.9% for each of oxytetracycline and trimethoprim-sulphamethoxazole, 51.6% nalidixic acid, 37.5% nitrofurantoin and 32.6% chloramphenicol.

Multiple antibiotic resistance to at least 2 different antibiotics (EUCAST, 2000) was frequent in the 3 farms and reached 65.5% for CF1, 90% for CF2 and 78% for CF3. Cross-resistance level was the highest for 2 different antibiotics in CF1, for 3 different antibiotics in CF2, and for 5 different antibiotics in CF3 (Fig. 2). Simultaneous resistance to the 6 tested antibiotics was mainly observed in *Enterobacter gergoviae* species (4 isolates in CF2 and 1 isolate in CF3). Among the 92 selected isolates, 73 were multiresistant. A total of 17 multiple antibiotic resistance profiles were recorded and the 7 most common are shown in Table 2. More than 78% of the multiresistant isolates fell within these 7 profiles (57 isolates out of 73). The major profiles included OTC-SXT-NA, AM-OTC-SXT-NA, and

Table 2
The seven most common antibiotic resistance profiles encountered in 73 multiresistant isolates from catfish farms

Antibiotic resistance profile	Bacterial species	Number of isolates	% isolates per profile
OTC, SXT	*Escherichia hermanii*	2	6.8
	Escherichia coli	3	
OTC, SXT, NA	*Escherichia hermanii*	1	15.1
	Xenorhabdus luminescens	9	
	Enterobacter agglomerans	1	
AM, OTC, SXT, NA	*Pseudomonas pseudomallei*	6	17.8
	Vibrio metschnikovii	2	
	Escherichia hermanii	3	
	Vibrio parahaemolyticus	1	
	Pseudomonas cepacia	1	
AM, FT, OTC	*Vibrio metschnikovii*	5	9.6
	Proteus vulgaris	1	
	Pragia fontium	1	
AM, C, OTC, SXT	*Escherichia coli*	5	6.8
AM, C, FT, OTC, SXT, NA	*Enterobacter gergoviae*	5	8.2
	Serratia plymuthica	1	
AM, C, FT, SXT, NA	*Pseudomonas cepacia*	10	13.7
Total		57	78.1

AM, ampicillin; C, chloramphenicol; FT, nitrofurantoin; NA, nalidixic acid; OTC, oxytetracycline; SXT, trimethoprim-sulphamethoxazole.

Table 3
MAR index for catfish farms (CF1, CF2, CF3)

Fish farm	Total isolates	MAR index[a]
CF1	29	0.36
CF2	40	0.57
CF3	23	0.62

MAR, multiple antibiotic resistance.
[a] According to Krumperman (1985).

AM-C-FT-SXT-NA which occurred respectively in 15.1%, 17.8% and 13.7% of bacterial isolates ($n = 92$). Considering these 7 most common profiles, most oxytetracycline -resistant isolates (47/57) were also resistant to trimethoprim-sulphamethoxazole (40/47 = 85%). The MAR index was determined for each farm (Table 3).

4. Discussion

4.1. Feeding practices for Pangasius sp. aquaculture in Mekong Delta (Viet Nam)

Home-made feed is still a common practice for *Pangasius sp*. cage and pond culture in Mekong Delta. It still accounts for more than 50% of total Mekong catfish production in Viet Nam. Home-made feeding, of which trash fish remains the main protein source, raises more and more constraints due to the trash fish market. As having many advantages in terms of feed efficiency, environmental management and availability, floating pellet feed is becoming more encouraged to use for catfish culture. A survey (Le Thanh Hung, Huynh Pham Viet Huy, Nguyen Thi Thanh Truc, & Lazard, 2006) conducted in three locations of Mekong Delta where catfish culture is the most developed (Chau Doc, Long Xuyen and Can Tho-Vinh Long) puts into evidence a strong diversity of feeding practices along the Mekong River. Roughly, it appears that home-made feed is mainly developed in the most ancient places where catfish culture is carried out (Chau Doc). In the two other locations, artificial feeding is dominant but in all the investigated areas both feeds are used, especially pellet feed during the first grow-out month and sometimes also during the last month in order to enhance the growth of fish just after the transfer from nursery to ponds or cages and to improve the quality of the flesh.

It has also been assessed that fish farmers could introduce feed additives at low concentration in order to improve the feed quality for a better fish health and growth (vitamin C, mineral premix, lysine, methionine, antioxidant, probiotics).

The economic survey concerning the catfish farms of the three locations showed that the cost of disease prevention and treatment, concerning mainly the purchase of antibiotics, accounted for more than 5% of total costs in the case of farms using home-made feed (cage and pond) and between 2% and 4% for farms using pellet feed. These data show clearly that there is still a wide use of antibiotics for growing catfish in cages even when using pellet feed. No information could be collected related to the use of antibiotics by industrial feed meals.

4.2. Antibiotic resistance

The microbial composition of the isolated bacteria was consistent with the literature (Al-Harbi & Uddin, 2004; DePaola, Peeler, & Rodrick, 1995; Spanggaard, Jorgensen, Gram, & Huss, 1993). Many of the organisms are ubiquitous in the aquatic environment (Table 1). Nevertheless, the presence of *Vibrio sp.* isolates is uncommon in freshwater aquaculture. This contamination is probably originated from the trash sea fish used as a main component of home made feed. It is important to note that the raw material was inappropriately conditioned and it arrived spoiled at the farm, which certainly increased the risk of contamination by *Vibrio sp*. This finding calls for further investigations since *Vibrio sp.* may cause fish diseases. On the other hand, the absence of *Aeromonas sp.* which is normally found in such an environment (McPhearson, DePaola, Zywno, Motes, & Guarino, 1991; Schmidt, Bruun, Dalsgaard, Pedersen, & Larsen, 2000), might be due to our use of a non selective medium (PCA) for the arbitrarily bacterial selection. The high recovery of members of the *Enterobacteriaceae* family in CF2 having the highest resistance rate to oxytetracycline (Fig. 1) may be due to a specific treatment that promotes enterobacteria, as observed by DePaola et al. (1995) with oxytetracycline-medicated feed. These authors showed that selected *E. coli* from catfish ponds in the USA were all susceptible to trimethoprim-sulphamethoxazole, chloramphenicol, and nitrofurantoin, and only 11.1% were resistant to ampicillin. These results contrasted with our findings, except for those on nitrofurantoin which were similar. On the other hand, McPhearson et al. (1991) found 24% and 100% of 38 *E. coli* isolates from catfish that were respectively resistant to nitrofurantoin and oxytetracycline. Nitrofurantoin and chloramphenicol correspond to two antibiotics forbidden under European Union regulation (no maximum residue limit has been established in animal-derived foods). Resistance frequencies found in our work for chloramphenicol were lower than in the study by Michel, Kerouault, and Martin (2003) who found 80% chloramphenicol-resistance among fish bacteria (brown trout, Atlantic salmon, brook trout and its hybrid). The nitrofurantoin-resistance rates were higher than those reported for fish isolates in the literature (Castro-Escarpulli et al., 2003; Liu, Lee, & Chen, 1997; Teophilo, dos Fernandes Vieira, dos Prazeres Rodrigues, & Menezes, 2002).

High multiresistance incidence similar to or higher than those found in this study has been reported in aquatic environments (McPhearson et al., 1991; Miranda & Zemelman, 2002; Vivekanandhan, Savithamani, Hatha, & Lakshmanaperumalsamy, 2002). The high recovery of multiresistant isolates (Fig. 2) may reflect an adaptation of bacteria to the "fluctuating antibiotic environment" as proposed by Baquero, Negri, Morosini, and Blazquez (1998). In response to this antibiotic pressure, the selected bacterial

strains are those which have multipurpose or multiple mechanisms of survival. Actually, several efflux pumps associated with increasing levels of multiple resistance to tetracycline, chloramphenicol, ampicillin, nalidixic acid and rifampin (Okusu, Ma, & Nikaido, 1996), to tetracycline, chloramphenicol and quinolones (Alonso & Martinez, 1997) have been characterised in Gram-negative bacteria. The MAR index values indicated that the three farms corresponded to high-risk exposed-antibiotic sources (Table 3). Actually the main antibiotics used in catfish farms and nurseries in the Mekong River Delta include β-lactamin, quinolone, aminosid, sulfamid, tetracycline but others combinations are used too according to the own experience of the farmers for disease prevention and treatment (Phuong, Oanh, Dung, & Sinh, 2005). These authors have tested the resistance of 123 bacterial isolates from water, sediment and different fish farms (catfish, tilapia, common carp and gouramy) in five provinces of the river. The results showed that 90% of the isolates were resistant to tetracycline, 76% to ampicillin, 100% to chloramphenicol, 65% to nitrofurantoin and 89% to trimethoprim-sulphamethoxazole which are very high scores as well. The intensive farming practices, the use and abuse of different drug families, and the use of broad-spectrum molecules contribute to the antibiotic pressure in the river. The literature has shown that the impact of farming practices extends beyond the individual farm environment (Schmidt et al., 2000). On the other hand, once acquired, resistance determinants could be maintained within the bacterial population even in the absence of the corresponding antibiotic (Chiew, Yeo, Hall, & Livermore, 1998).

5. Conclusion

In conclusion, the results of this study showed that antibiotic resistance and multiresistance occurred at high level among indigenous bacteria isolated from catfish farming in the Mekong River Delta. The economic survey has shown that antibiotic purchase represents a high cost for home made feed. Furthermore, MAR index values indicated a high-risk exposed-antibiotic sources for these farms. It appears thus that antibiotic resistance patterns should be monitored as a routine to improve the design and the implementation of efficient chemotherapy strategies. This could be included in the HACCP (hazard analysis critical control point) system procedures to achieve a global food control strategy. In addition, alternatives to antibiotics should be explored such as the development of vaccination as experienced for the Norwegian aquaculture (Lillehaug, Lunestad, & Grave, 2003; Lunestad & Grave, 2005). and that should be a lesson for catfish industry development to ensure its sustainability and food safety.

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ORIGINAL RESEARCH

Chemiluminescent and bioluminescent assays as innovative prospects for mycotoxin determination in food and feed

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ABSTRACT: Mycotoxin contamination of food and feedstuffs is among the top priorities for human and animal safety. The currently used techniques for mycotoxin determination, either chromatography or ELISA, are unsuitable for routine in-field assessment. There is an urgent need for other accurate, simple and cost-effective techniques that can be used as a screening tool for a rapid estimation of mycotoxin contamination in commodity lots. This paper reviews the literature on the use of chemiluminescence (CL) and bioluminescence (BL) assays for direct or indirect mycotoxin assessment. The chemiluminescence immunoassays, adenosine triphosphate (ATP) bioluminescence and bioassays are reviewed and their advantages and limitations discussed. These techniques used in food testing and the pharmaceutical industry offer promise as rapid techniques for mycotoxin determination. Chemiluminescence and bioluminescence bioassays are the most innovative alternatives to the conventional techniques used for mycotoxin determination in food and feed. Copyright © 2004 John Wiley & Sons, Ltd.

KEY WORDS: mycotoxin, bioluminescence, chemiluminescence, bioassay, food testing

INTRODUCTION

Mycotoxin contamination of food and feedstuffs is among the top priorities for human and animal safety. Consumers are more and more concerned by public health-related issues and show high preoccupation about the risks associated with human exposure to mycotoxins. Indeed, mycotoxins are fungal metabolites that have been shown to be carcinogenic, mutagenic, teratogenic and immunosuppressive (1). Furthermore, mycotoxins have attracted worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and both domestic and international trade (2, 3).

The main mycotoxins currently considered of importance include the aflatoxins, the tricothecenes, zearalenone, the fumonisins and ochratoxin A. They can occur in various commodities, such as cereal grains, pulses, nuts, milk and dairy products, coffee and wines (4, 5). The control of mycotoxins is currently pursued through quality and regulatory procedures. Limits of these substances in traded goods are becoming more and more restrictive. The techniques currently most used for mycotoxin determination are: (a) chromatography, including high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and gas chromatography–mass spectrometry (GC–MS); and (b) ELISA techniques. Considering the limitations of these techniques (see next section), there is still an urgent need for other accurate, simple and cost-effective techniques that could be used as a screening tool for a rapid estimation of mycotoxin contamination in commodity bulks. Luminescence assays currently used in food testing and the pharmaceutical industry (6) could be promising as rapid and sensitive techniques.

This article reviews the bioluminescence (BL) and chemiluminescence (CL) methods that have been investigated for mycotoxin analysis and discusses their advantages and limitations for the determination of mycotoxins in food and feed.

STATE OF THE ART ON ANALYTICAL METHODS FOR MYCOTOXIN DETERMINATION

The assessment of contamination is based on the analytical determination of mycotoxins. Conventional methods include: high-performance liquid chromatography (HPLC), with either fluorescence or diode array detection; thin-layer chromatography (TLC); and gas chromatography coupled to mass spectrometry (GC–MS) or electron capture detection (GC–ECD). These methods are used for determination of different types of mycotoxins, such as aflatoxins, ochratoxin A, fumonisin and deoxynivalenol (DON) in different commodities, such as coffee (7–9), cereal and legume grains (10, 11),

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black pepper (12), wine and beer (5, 13, 14) and cheese (15). However, these methods need sophisticated equipment and trained analysts, and the procedure for mycotoxin extraction from the sample must be adapted according to the type of mycotoxin and food matrix to be tested. Progressively, improvements were made to the chromatographic methods through the introduction of clean-up procedures by use of immunoaffinity columns and post-column derivation, but this has increased the cost of analysis. Traditional methods involve lengthy extraction procedures, expensive chemical clean-up and require the handling of hazardous materials, all factors which make such tests unsuitable for routine use in the food industry or in a farm setting (16).

The availability of rapid diagnostics for detection of toxins in the food chain is an area which has developed rapidly in the last 10 years, predominantly in response to legislative requirements (17). This orientated analytical mycotoxin assessment towards the application of enzyme-linked immunosorbent assays (ELISA). Most commercial ELISAs for mycotoxins rely on competition between the toxin from the sample with a labelled toxin (such as a toxin–enzyme conjugate) for a limited number of antibody-binding sites. The greater the amount of toxin present in the sample, the lower the binding of the labelled toxin and the lower the signal generated by the assay (18–20).

Research was initially performed on experimental matrices, such as mycotoxin standards, urine or blood serum samples, or extracts from pure mould cultures. Later on, ELISA techniques were adapted for mycotoxin determination (21–25). Many commercial kits are now available, such as Ridascreen kits for aflatoxin, fumonisins, DON and zearalenone (R Biopharm Ltd), Aflatox Cup (International Diagnostic System) and Aflatoxin B_1 test (Cite Probe). They are simple and easy to use, with a moderate to high cost, according to the context of use. However, ELISA techniques have been shown to be less accurate and sensitive than conventional chromatographic assays. Very few correlations were found between the two types of techniques. In addition, false positive or false negative results often occurred with ELISA because of cross-reactions between molecules or interferences with the antibody reagents. They are thus considered to be suitable for qualitative assessment or for sample pre-screening but not for quantitative determination. It is also recommended to use the ELISA techniques for the foods they were developed for. It should be pointed out that the ELISA techniques are nowadays mostly used by industries for rapid monitoring, whereas chromatographic methods are still used in laboratories for investigation purposes.

Fluorescence polarization immunoassays (FP) have been developed in a laboratory context for aflatoxin (26) and DON (27) determination in grains. These assays are based upon the competition between free aflatoxin and an aflatoxin–fluorescein tracer for an aflatoxin-specific monoclonal antibody in solution. FP assays have two important differences from ELISA: the detection does not involve an enzymatic reaction, and separation of the bound and free label is not required. As a result, FP assays do not require the wash step essential to many ELISAs, and they do not require waiting for an enzyme to produce a coloured product. Following the same approach, the enhancement of luminescent capacity of mycotoxins through immunoenzymatic assays could be used as an indicator for contamination assessment.

Biomolecular techniques, such as polymerase chain reaction (PCR), are emerging as promising tools for mould detection, rather than mycotoxin determination (28). However, these techniques require expensive investment and trained analysts and can only serve for detecting the responsible coding genes of mycotoxin-producing moulds but not for routine in-field assessment of contamination, especially in developing countries.

CL IMMUNOASSAYS

Chemiluminescence is based on a chemical reaction that can be described as follows:

$$A + B = C^* + D = C + \text{light}$$ (29), where * indicates an electronically excited state.

In aqueous solution, the most frequently used CL compound is luminol (5-amino-2,3-dihydrophtalazine-1,4-dione or its derivatives, such as isoluminol). Luminol reacts with H_2O_2 in the presence of a catalyst (metal or metal-containing compound or enzyme) in alkaline solution to yield 3-aminophthalate in an excited electronic state, which returns to the ground state with the production of light. However, the intensity of the light signal is low and of short duration. To amplify and prolong the signal, a compound known as 'enhancer' (e.g. 4-iodophenol) is added to the reaction medium ('enhanced chemiluminescence'). The enhanced CL reaction is one of the most sensitive and rapid detection methods in medical and analytical biochemistry (30, 31).

This luminescent reaction can be used for the detection of antigen–antibody binding at the final stage of an immunoenzymatic assay. Such a solid-phase chemiluminescent immunoassay (CIA) is described below (Fig. 1). This technique was used to analyse ochratoxin A and the results were compared to a conventional ELISA test [OcA–HRP (horseradish peroxidase) conjugate] (32). The CL compound used was ABEI (N-4-aminobutyl-N-ethyl-isoluminol) and this was conjugated to the mycotoxin antigen (OcA–ABEI).

As shown in Fig. 1, the assay protocol is based on the following steps:

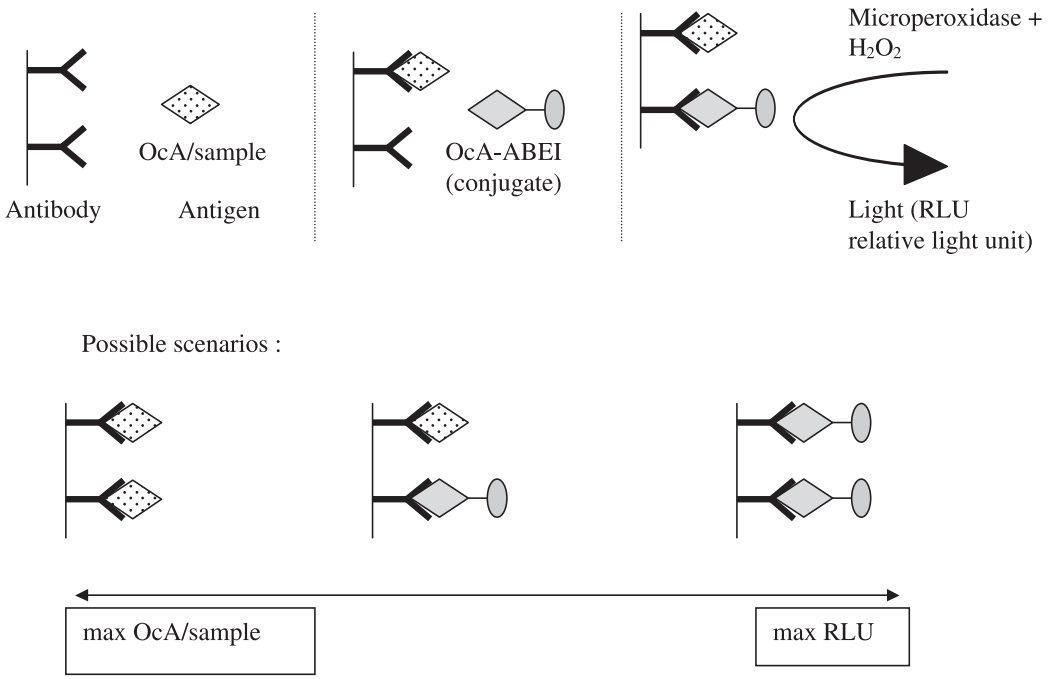

Figure 1. Solid-phase chemiluminescent immunoassay, as described by Kim *et al*. (32) for ochratoxin A (OcA). ABEI, N-4-aminobutyl-N-ethyl-isoluminol.

1. Addition of the sample or standard solution to the antibody-coated tube.
2. Addition of the tracer OcA–ABEI for the CIA assay.
3. The mixture is incubated at 37°C/2 h.
4. Removal of the solution and 3 times washing of the antibody-bound fraction with phosphate buffer.
5. Addition of NaOH (5 mol/L) and incubation at 60°C/1 h.
6. Injection of the microperoxidase solution and diluted H_2O_2 to the assay tube in the luminometer.
7. Integration of the signal for 4 s.

There was an excellent correlation (r = 0.996) obtained between the results of ELISA (OcA–HRP) and those of the CIA (OcA–ABEI) and this shows that the CL technique could replace conventional ELISA. The detection threshold for the CL immunoassay was 20 pg ochratoxin A/tube. This sensitivity is similar to that obtained with ELISA for ochratoxin A analysis in wheat (33). Known quantities of ochratoxin A were added to corn samples which were analysed before and after this step. In these conditions, less than 70% of the added material was recovered. This loss may probably arise from the extraction procedure, which constitutes a major problem for mycotoxin food determination. According to this observation, this CL immunoenzymatic assay cannot yet be used in routine testing for agricultural commodities.

A similar limitation has been observed by Wittmann and Schreiter (34), who tested a CL ELISA to quantify a herbicide, terbuthylazine (*s*-triazines class) in soil samples. This interesting study used a peroxidase label, and the label was detected using the enhanced CL reaction (detection reagents: luminol + *p*-iodophenol + hydrogen peroxide in NaOH/borate buffer, pH 8.5).

The thresholds of detection obtained by the various methods were as follows:

- ELISA (HRP label): 0.3–3 µg/L.
- ELISA (alkaline phosphatase label) detected by reflectance: 3–300 µg/L.
- ELISA (with HRP label) detected by CL: 0.05–10 µg/L, showing a lower detection limit and a larger range of measurement.

The ELISA with luminescence detection showed a superior measuring range but failed in the measurement of the soil samples collected from the environment. Indeed, the authors analysed samples from the environment according to three methods: (a) gas chromatography; (b) reflectance immunoassay (alkaline phosphatase); and (c) CL immunoassay (HRP). The first two techniques gave similar results, whereas the CL ELISA type using an HRP label revealed several false positive results.

It can be presumed that either a matrix effect, an interference affecting the detection method or antibody cross-reactivity could be the potential source of the overestimation. However, the source of the discrepancy was not determined. The CL immunoassay format must therefore be improved before it can be used as a routine field testing method.

In the context of enhanced CL assays, it is important to note that buffers are not inert and should be considered as a crucial fourth component of the HRP-enhanced CL light-generating system (35). Cercek *et al.* (36) shows that, at the same pH and HRP concentration, the magnitude of the light signal (RLU) is significantly influenced by the reagent buffer composition, and could increase up to 10-fold, e.g. at pH 7.3 and HRP concentration 4 fmol/40 µL assay, the glycine buffer signal is 10 times higher than that obtained with tricine buffer. At pH 8.5 and 1.6 fmol HRP/40 µL assay, the borax buffer gives the highest magnitude, which is 10-fold higher than with glycylglycine buffer.

The CIA method has been used by Kang *et al.* (37) to determine the production, under artificial conditions, of ochratoxin A by several fungi strains (from the genera *Aspergillus*, *Penicillium*, *Paecilomyces*) isolated from Korean traditional fermented soybean foodstuffs (Maeju, Dwangjang and Kangjang). The method was accurate in the 20 pg/assay range with a 90% recovery in laboratory cultures, but determination of mycotoxin directly from the fermented soybean foodstuff was not indicated.

ATP BIOLUMINESCENCE

Another alternative to the direct determination of mycotoxins in food is the detection of fungi in the sample. We are aware that the presence of fungi is not a direct determination of the presence of mycotoxins but it could serve as a screening test to distinguish potential mycotoxin-producing food lots. ATP bioluminescence has been mostly used for determining yeast or bacterial contamination in food (38–44). It is a rapid technique, based on the reaction between microbial ATP and firefly luciferase to produce luminescence. The microbial ATP is extracted with acid, organic solvent or detergent after the removal of somatic ATP (from non-microbial cells, if any) together with any free ATP using ATPase. Luciferase is then added and the emitted light is measured using a luminometer.

The amount of light may be directly related to the microbial number (45, 46) but both difficulties of extracting ATP from moulds and distinguishing it from the bacterial ATP could explain why this method is not recommended for moulds (47). Kaspersson *et al.* (48) measured the total ATP contents by BL in several foodstuffs during storage and correlated it to the count of bacteria, yeasts and moulds. They showed that the best correlation was obtained with bacteria and the worst on food contaminated by moulds.

BIOASSAYS

Tests using bacteria as indicators are widely used to identify the toxicity of chemicals or commercial products, and environmental pollution (49, 50). Both CL and BL have been investigated to assess the effects of mycotoxins on biological systems. We review the principles of these different CL- and BL-based methods to show how they could be innovative for toxicological assays applied to mycotoxins.

Nishimoto (51) tested the toxicity of toxins from *Fusarium* species on viable human cells (HuH-6KK). This CL assay was not intended to determine mycotoxin concentrations, but instead to evaluate their toxic effect on cellular cultures. The cells (HuH-6KK, NIH3T3, PC-3) were trypsinized and used to prepare 2×10^4 cells/well (500 µL) in 24-well tissue culture plate and the cells mixed with the *Fusarium* toxins (NIV, DON, FX) and incubated for 48 h (Fig. 2). The viability was then

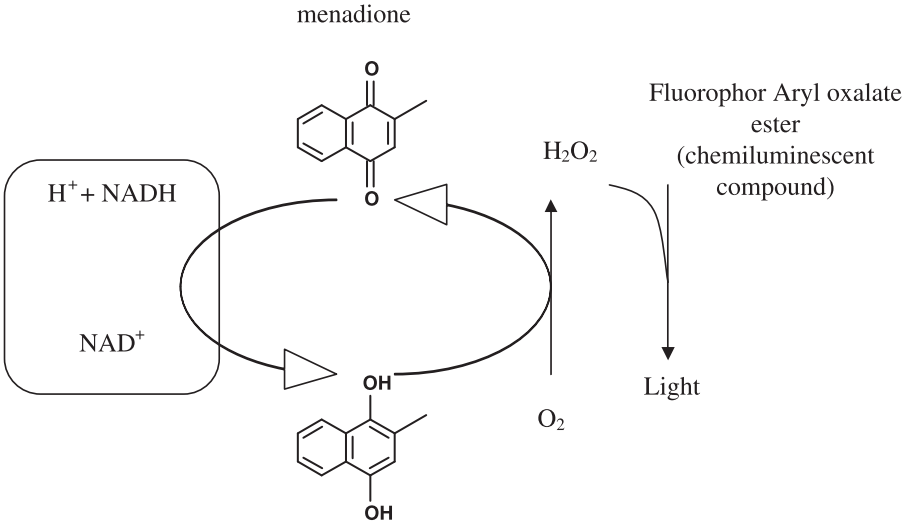

Figure 2. Mechanism of the production of H_2O_2 catalysed by exogenous menadione in cell culture (51).

assessed by the CL technique, which consists of adding a solution of menadione/ethanol into the medium for incubation at 37°C for 10 min. After that, 50 µL CL reagent [10 mg bis(2-(3,6,9-trioxadecanyloxycarbonyl-4-nitrophenyl) oxalate + 1 mg pyrene in 10 mL acetonitrile] was added and the total emitted light measured by integration. The results show that the production of H_2O_2 through the mechanism described above is proportional to the cellular enumeration. Based on these findings, human cell cultures (HuH-6KK) were monitored in presence of various *Fusarium* mycotoxins—nivalenol (NIV), deoxynivalenol (DON) and fusarenon-X (4-acetylnivalenol, FX). The cellular activity was measured by CL after 24 h of incubation and was compared to the conventional methods: (a) neutral red inclusion assay; and (b) MTT [3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay. The viability curves showed a perfect correlation between these three methods. Indeed, the CL assay was superior in speed and sensitivity for the detection of cytotoxicity, the intensity of which was observed in the following order: FX > DON > NIV. This assay makes possible the assessment of the cytotoxic effect of *Fusarium* toxins on human cellular cultures (HuH-6KK). Similar results were obtained on mouse fibroblasts (NIH3T3) and human prostate cancer cells (PC-3).

Another toxicity test is based on the natural genetic BL potential of bacteria due to specific genes called '*lux* genes'. The bacterial luminescence reaction (Fig. 3) is catalysed by luciferase and can be summarized as follows (52):

$$RCHO + FMNH_2 + O_2 = RCOOH + FMN + H_2O + h\nu$$

Luciferase is an enzyme composed of two subunits, α and β, encoded by genes *luxA* and *luxB*, respectively (53). These *lux* strains belong to genera such as *Vibrio*, *Photobacterium*, *Xenorhabdus* and *Alteromonas*. Two species, *Vibrio fisheri* and *V. harveyi*, have been investigated intensively. In both, *lux* genes are organized in an operon together with other genes involved in the BL reaction. Expression of the *lux* operon in both strains undergoes a specific regulation, called 'quorum sensing' (54). In the presence of inhibiting substances such as antibiotics, other antimicrobial agents, bacteriophages, or toxic substances such as mycotoxin, the reduced numbers of bacteria can be detected by BL through the determination of effective concentration EC_{50}, which corresponds to the mycotoxin concentration that causes a 50% reduction of bioluminescence.

The use of bacterial BL as a toxicological assay for mycotoxins was investigated with *V. fisheri* (formerly *Photobacterium phosphoreum*). The protocol is described by Bulich and Isenberg (55). It uses the Microtox*™ method (registered trademark of Beckman Instruments) with a freeze-dried luminescent bacterium, *V. fisheri*. The sample is adjusted to 2% NaCl and then diluted using the diluent provided (osmotic protection to the marine microorganism). The luminescent bacteria are hydrated with the reconstitution solution provided, cooled to 3°C, and aliquots are transferred to cuvettes containing 0.5 mL diluent equilibrated to 15°C. Initial light measurement is made for each cell suspension. Sample dilutions and the control are then added and the light is again measured after 5 min for each cuvette. Using this method, Yates and Porter (56) studied the effect of different mycotoxins (patulin, penicillic acid, citrinin, zearalenone, ochratoxin A, aflatoxin B1, rubratoxin B). Bioluminescence determinations were made with the Microtox* analyser at 5, 10, 15 and 20 min after addition of the toxin to the microbial suspension. All these mycotoxins induced an EC_{50} (µg/mL) after 5 min of incubation ranging from 7.51 µg/mL for patulin to 31.79 µg/mL for rubratoxin B. The inhibitory action of all mycotoxins approached the maximum effect by 15 min. These assays depend much on the nature of the mycotoxin, the pH, the temperature,

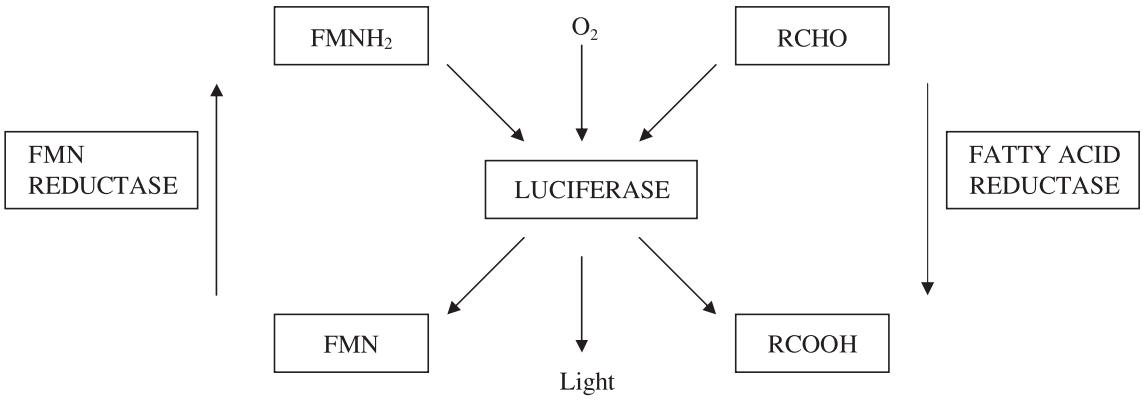

Figure 3. Mechanism of bacterial bioluminescence: FMN, flavin mononucleotide; RCHO, long-chain aldehyde; RCOOH, long chain fatty acid (52).

the age of the bacteria and the time exposure. With the exception of zearalenone, the longer the incubation time, the less the concentration of toxins required to induce a EC_{50} of *P. phosphoreum*.

When increasing the pH of the assay suspension from 6.0 to 8.0 (testing at pH 6.0, 6.5, 7.0, 7.5 and 8.0), the toxicity of zearalenone and penicillic acid decreased and, inversely, the toxicity of patulin increased. Aflatoxin B1 demonstrated its greatest toxicity at pH 7.5. Each mycotoxin showed its greatest toxicity at a different pH and temperature combination: patulin, pH 8.0/30°C; penicillic acid, pH 6.5/25–30°C; zearalenone, pH 6.0/10–15°C, and aflatoxin B1, pH 7.0–7.5/20–30°C. This study showed that optimizing pH and temperature for each mycotoxin enhanced the sensitivity of the bacterial BL assay (57).

These results demonstrated a reliable short-term method for assessing the toxicity of mycotoxins. The advantage of this system is its sensitivity and simplicity. Such a procedure may serve to discriminate food samples, but it should be emphasized that the food sample extract (corn, maize or wheat) might contain other fungal metabolites or pesticide residues accumulated in the grains and which could also have an inhibitory effect on the bacterial activity.

Kratasyuk *et al.* (58) developed a luciferase-based BL test for monitoring wheat grain (*Triticum vulgare*, type IV) infection with *Fusarium*. The test kit 'NADH-KRAB' contains: (a) NADH-reagent (lyophilized preparation of luciferase and NADH:FMN oxidoreductase); (b) 0.2% alcohol solution of tetradecanal; and (c) FMN and NADH. The results show that infected grain extract inhibits bioluminescence, and that the inhibition increases when the content of infected wheat grains increases in the mixture sample (consisting of intact and infected grains). The maximum inhibitory effect was observed when the content of infected grains was 0.5–1.5%, which corresponds to a DON (the main mycotoxin produced by *Fusarium*) concentration of 0.5–1.5 mg/kg. The authors proposed that this method measures a total toxicity rather than just the mycotoxin effect. They proposed to use it as a rapid screening method for rejecting infected grain batches in production.

CONCLUSION

We should emphasize that mycotoxin management is more and more switching from an expensive wasteful end-point testing/segregation towards an integrated, systematic and cost-effective approach throughout the whole agrifood chain. This increases the need for analytical tools that are accurate, cost-effective and suitable for *in situ* routine control. While a range of kits exist today, there are still limitations to their use *in situ* and the economic costs can still deter use, mainly in developing countries, even when they are heavy exporters of commodities. This article reviews the literature on BL and CL techniques for mycotoxin determination in food and feed. The CL immunoassay and BL-based bioassays showed good sensitivity and accuracy in laboratory studies. These methods constitute an innovative alternative to conventional techniques for mycotoxin analysis. However, they need to be improved before they can be applied in large-scale food production testing.

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High efficiency styrene biodegradation in a biphasic organic/water continuous reactor

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Abstract. Styrene was degraded as sole source of carbon and energy by a selected bacterial community in a two-phase aqueous-organic medium (80%:20%, vol/vol). Silicone oil was used to solubilize styrene, which is sparingly soluble in water and to prevent its toxicity toward microorganisms. Preliminary studies with the mixed population in batch cultures indicate that the specific activity and the maximum growth rate at optimal pH 6.0 were 46 $mg \cdot g^{-1} \cdot h^{-1}$ and 0.15 h^{-1}, respectively. In pH-regulated chemostat cultures, styrene was degraded at dilution rates ranging from 0.05 to 0.20 h^{-1}. Kinetic parameters and the proportion of each strain in the mixed culture were followed. At 0.20 h^{-1}, only one strain as compared to four initially present, remained in the medium. This strain *Pseudomonas aeruginosa*, degrades styrene with a specific activity of 293 $mg \cdot g^{-1} \cdot h^{-1}$. Such results could lead to industrial treatment of waste gas or water polluted with styrene.

Introduction

Styrene is essentially used in the chemical industry for the manufacture of synthetic polymers such as polystyrene, styrene-acrylonitrile or styrene-butadiene rubbers. Styrene can cause severe damages (Leibman 1975). The ways of its absorption in industrial exposure are usually pulmonary and percutaneous. The toxicity of styrene in mammalian organisms is due to its oxidation into styrene oxide, which represents the active metabolite. The recommended threshold limit value (TLV) is 100 ppm. The ambient atmosphere of styrene manufacture is thus continuously refreshed, yielding significant air flows to be treated. Styrene is usually eliminated in the latter through thermic processes. Conversely, no biological treatment seems to be used on an industrial scale, whereas styrene has been shown to be fully degraded by selected microorganisms.

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Its aerobic microbial metabolism proceeds via the formation of 2-phenylethanol and phenylacetic acid, as demonstrated by several studies using mixed or pure culture (Sielicki et al. 1978; Baggi et al. 1983). Shiraï and Hisatsuka (1979) reported the role of styrene oxide as an intermediate metabolite in the oxidation of styrene to 2-phenylethanol by *Pseudomonas* 305-STR-1-4. Hartmans et al. (1989, 1990) discussed, with more details, the enzymes involved in the degradation of styrene oxide and 2-phenylethanol by *Xanthobacter* 124X. These investigations suggest a second mechanism whereby the degradation of styrene occurs by initial oxidation of the aromatic ring. Other studies on the biodegradation of aromatic compounds such as *n*-butylbenzene, isobutylbenzene and isopropylbenzene, suggest the co-existence of two different pathways, one through oxygenation of the side chain and the other through hydroxylation of the benzene nucleus (Jigami et al. 1979).

In this paper, we report the degradation of styrene by a mixed population of microorganisms able to utilize this molecule as the unique source of carbon and energy. As styrene was sparingly soluble in water and toxic toward microorganisms, the fermentation was within a liquid-liquid biphasic system (Lebeault 1990). A silicone oil was used as the organic phase to solubilize styrene and to hinder its toxicity. The selection of microorganisms and the optimisation of the culture pH were performed in batch fermentation. The degradation kinetics and the microbiological behaviour of the selected bacterial community were then studied in a chemostat reactor.

Materials and methods

Microorganisms and growth conditions. In a preliminary industrial work, a mixed population of bacteria degrading a styrene and acrylonitrile mixture was selected by the enrichment culture technique. In order to study the biodegradation of styrene alone, those microorganisms were then grown and subcultured in a basal salts mineral medium (BSM) supplemented with 20% (v/v) silicone oil 47 V20 (Rhône-Poulenc, Lyon, France). Styrene, the sole carbon source, was introduced in the organic phase. The

BSM contained the following (per litre of distilled water): $(NH_4)_2SO_4$, 10 g; KH_2PO_4, 1.75 g; K_2HPO_4, 4 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.15 g; NaCl, 1 g; $FeCl_3$, 5.10–3 g; $MnCl_2 \cdot H_2O$, 1.10–3 g; $CuSO_4$, 1.10–3 g; Na_2MoO_4, 1.10–3 g; $ZnCl_2$, 1.10–3 g. Culture was carried out in a 2-l fermentor (Setric, Toulouse, France) in 1.2 l total working volume. The reactor was equipped with pH, temperature, air-flow (10 vvh) and agitation controls. All concentrations, including styrene concentration, are related to the total volume of both phases aqueous and organic.

Batch culture. The mixed culture (nine bacteria) was acclimated by an enrichment technique in the bioreactor at 30° C in six successive batches. The styrene concentration increased step by step from 1 g/l to 6 g/l. The pH was maintained around 7.0 with 5 M NaOH. Periodically the culture sample was plated onto agar plates to follow the composition of the growing population. The morphological differences between strains were discerned by: the colour and shape of the colony, Gram coloration of the strain and direct microscopic observation (motility).

After the selection period, a stable mixed population was obtained. It contained four different bacterial strains. Their identification was made with the Galerie API 20 NE system (Biomérieux, France). The optimal culture pH and the growth kinetics at the optimal pH were then determined in batch culture in the fermentor at 30° C. The initial concentration of styrene was 3 $g \cdot l^{-1}$.

Chemostat culture. The fermentor was fed at 6 g styrene $\cdot l^{-1}$ total volume for 100 days. The pH was controlled at 6.0 with 5 M NaOH. Seven steady states were carried out, corresponding to seven dilution rates ranging from 0.05 h^{-1} to 0.20 h^{-1}. The growth and kinetic parameters, as well as the strain composition of the mixed culture, were determined for each steady state, after at least four residence times for stabilization of the process.

Analytical methods. The organic and the aqueous phases of the sample were separated by ultracentrifugation at 25000 *g* for 30 min (Beckman L8.70) due to the high affinity of the microorganisms toward the oil phase. The styrene concentration was determined in the supernatant organic phase by flame ionisation detection gas chromatography (Intersmat IGC 121 DFL) with a 3% SP1500 Carbopack B column, under isothermic conditions: 200° C column and 260° C injector and detector.

The cells were harvested and dried at 110° C for 24 h to determine the dry weight biomass (g/l of total volume).

The pH and pO_2 of the medium were recorded using specific electrodes (Ingold).

After serial dilutions of the sample, 100 µl were plated onto agar plates to determine the percentage of each bacterial species.

Results

Batch culture

Styrene was solubilized in silicone oil to avoid microbial growth inhibition. Using the biphasic medium water/silicon oil, a stable mixed population able to utilize styrene as sole source of carbon and energy was selected. This bacterial consortium contained four different strains, identified as *Pseudomonas putida* (C1), *Achromobacter* sp. (C2), *Achromobacter* sp. (C3), and *P. aeruginosa* (C4).

Each strain was tested in an erlenmeyer flask to show its ability to degrade styrene (1 g/l). The results are summarized in Table 1. Only two strains (C1 and C4) were able to grow on styrene as the sole source of carbon and energy. The two others strains were probably involved in lower metabolism.

Under non-regulated pH conditions and at 10 vvh of aeration, both styrene and biomass concentrations were plotted as a function of time (Fig. 1a). The changes in pH and pO_2 during growth are represented in Fig. 1b. During styrene degradation, the pH dropped from 7.0 to about 4.0. The specific growth rate during the exponential phase was 0.06 h^{-1} and the average specific activity was 27 $mg \cdot g^{-1}$ dry cells $\cdot h^{-1}$.

The optimal pH for styrene degradation was determined in regulated pH batch cultures controlled by ad-

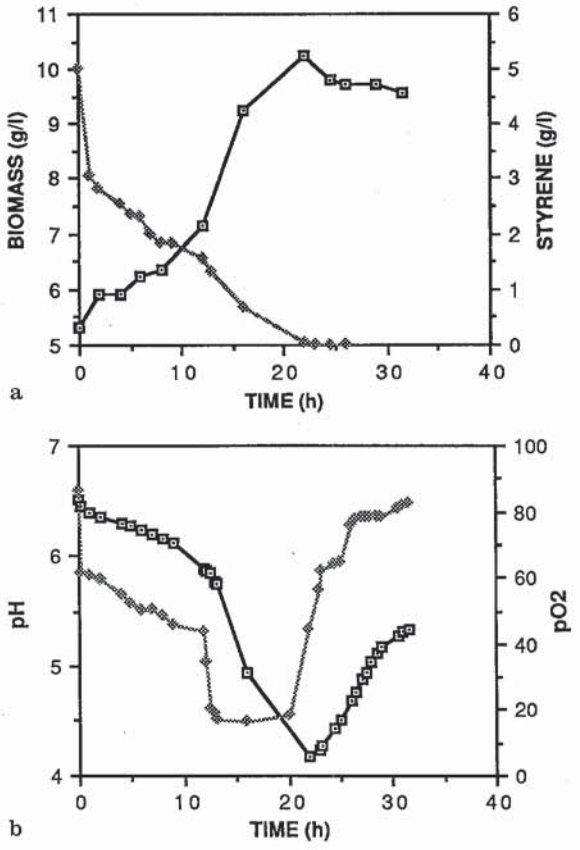

Fig. 1. a Changes in biomass (⊡) and residual concentration of styrene (◆) with time. **b** Changes in pH (⊡) and pO_2 (◆) with time

Table 1. Growth of mixed and pure cultures with or without an organic phase

Styrene (1 g/l)	Mixed culture	*Pseudomonas putida*	*Achromobacter* sp.	*Achromobacter* sp.	*P. aeruginosa*
Biphasic medium	+	+	−	−	+
Monophasic medium	−	−	−	−	−

+, growth; −, no growth

dition of 5 M NaOH. Figure 2 indicates that pH 6.0 was the optimal value for microbial growth. Under these pH conditions, styrene degradation was completely achieved in 13 h (Fig. 3). The specific growth rate increased up to 0.15 h^{-1} and the overall growth yield was 0.96 g dry cells·g^{-1} styrene. After exhaustion of styrene in the medium, the stationary step was reached, then the pO_2 recovered its initial value. This result explains the role of styrene as the growth-limiting substrate. The pO_2 parameter is a good indicator of styrene degradation. The specific activity and the biodegradation rate were respectively 46 mg·g^{-1}·h^{-1} and 230 mg·l^{-1}·h^{-1} (calculated from the first 13 h of growth).

Chemostat

Continuous culture offers the possibility of selecting microorganisms in a constant environment on the basis of their different growth rates. In our case on styrene, the dilution rate (D) was increased from 0.05 to 0.20 h^{-1}. The remaining culture became more and more efficient as the D increased. Actually, during this experimentation the specific activity increased linearly from 49 mg·g^{-1}·h^{-1} at 0.05 h^{-1} to 293 mg·g^{-1}·h^{-1} at 0.20 h^{-1} (Fig. 4). As the D increased, the biomass concentration decreased and the remaining styrene increased. This is shown in Fig. 5 where both biomass and percentage of styrene degradation are plotted as a function of D.

The relative proportion of each strain of the mixed culture varied during the experimentation (Fig. 6). The initial population contained four bacteria. At D = 0.12 h^{-1}, two strains (C2 and C3) were washed out. At D = 0.15 h^{-1}, only the C4 strain remained in the chemostat until 0.20 h^{-1}, which corresponds to a doubling time of 3.46 h. This last organism, which has been shown to be a gram-negative, motile rod, was oxidase and catalase positive. The colony has a green pigmentation. Based on these criteria and the Galerie API system, it was classified as *Pseudomonas aeruginosa*.

After centrifugation of the broth sample, the aqueous phase has a yellow colour. The formation of this coloration during growth with styrene was observed by Hartmans et al. (1989). Based on this indication, these authors demonstrated that styrene metabolism in *Xan-*

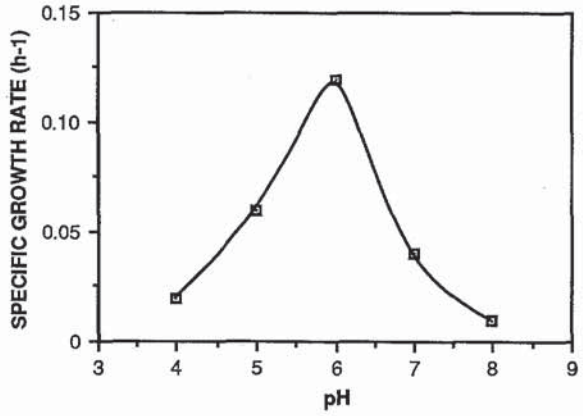

Fig. 2. Determination of the specific growth rate of the culture at different pH

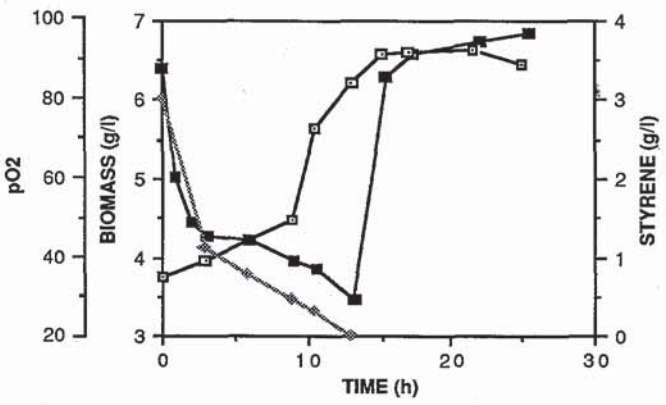

Fig. 3. Changes in pO_2 (■), biomass (⊡) and residual styrene concentration (◆) with time

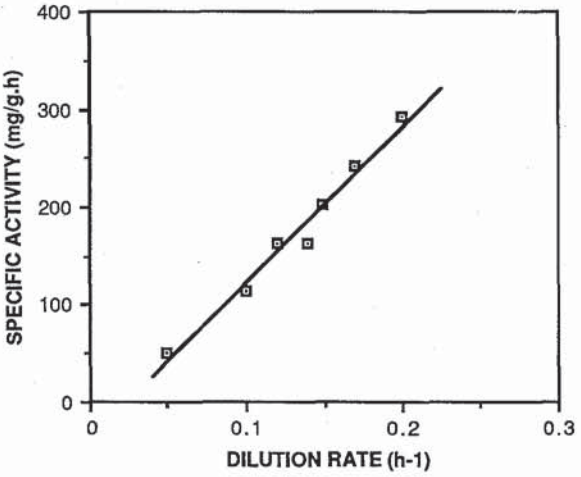

Fig. 4. Changes in the specific activity of the culture as a function of the dilution rate

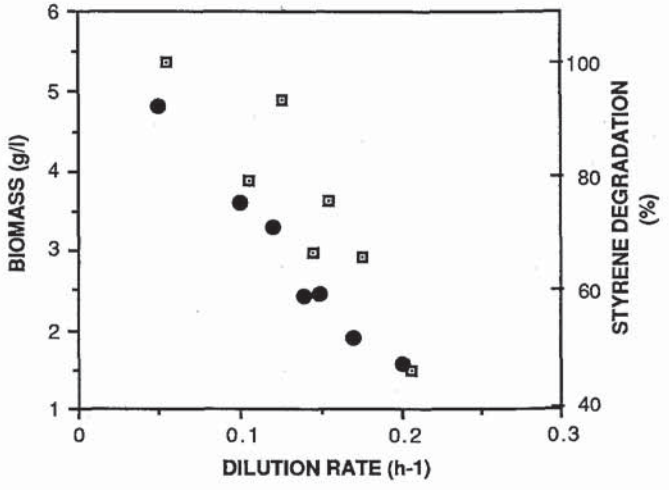

Fig. 5. Changes in biomass (●) and the styrene degradation rate (⊡) in the chemostat as a function of the dilution rate

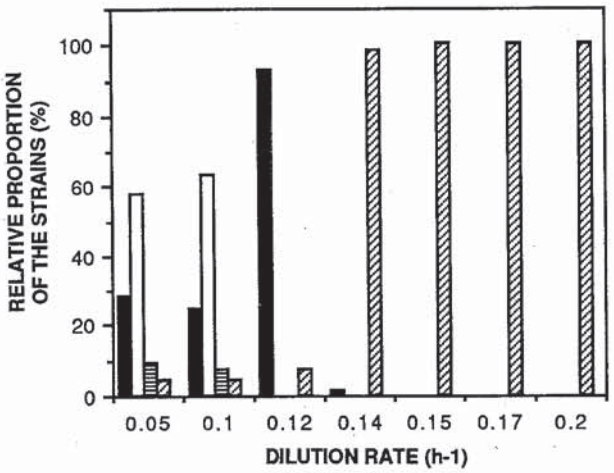

Fig. 6. Variation in the different strains (■, C1; □, C2; ▤, C3; ▨, C4) of the mixed culture during chemostat selection

thobacter sp. strain 124X has more in common with 1-phenylethanol than with styrene oxide metabolism.

Discussion

The utilization of an organic phase to enhance biodegradation of hydrocarbons has already been reported. Furuhashi et al. (1986) demonstrated that addition of *n*-hexadecane promoted the rate of styrene oxide production from styrene by *Nocardia corallina* B-276. In the absence of *n*-hexadecane, only a trace of styrene oxide was produced, due to substrate inhibition. In the case of epoxidation of 1-alkene with a chain length from six to nine carbons, the same strain was inhibited by the epoxide product; addition of *n*-hexadecane to the reaction system increases the rate of epoxidation of these substrates and the final product concentration. Dibutylphtalate was also used as a solvent by Bestetti et al. (1989) to circumvent styrene toxicity during *P. putida* growth. Actually two-phase aqueous-organic solvent systems can improve the performance of fermentation involving substrates or metabolites that are relatively insoluble in water or toxic.

In our work, no growth was observed in a control erlenmeyer flask containing mineral medium with 20% silicone oil and biomass. If the silicone phase was omitted, microorganisms were unable to oxidize styrene. Such results indicate that silicone oil is not attacked by microorganisms and was necessary to permit microbial styrene oxidation. It has been demonstrated that the lethal dose of styrene in aqueous medium is 70 $mg \cdot l^{-1}$. Using the biphasic system, the toxicity of this xenobiotic compound toward cells was avoided up to 6 $g \cdot l^{-1}$.

The selected bacterial population was able to grow on styrene as a sole carbon source without any additives, except minerals. Whereas multiple substrates are frequently used to improve the biodegradation of xenobiotic compounds by mixed cultures (Grady 1985), we needed no addition of co-substrate to facilitate adaptation to styrene. This result suggests that at low concentration in water phase, nutritional requirement for styrene degradation is minimal. This is confirmed by the high biomass yield during biodegradation (0.96 $g \cdot g^{-1}$). Styrene was mainly converted into biomass and few if any metabolites accumulated. The complete biodegradation (biomass, CO_2 and water) of the xenobiotic compounds is very interesting for making this process harmless for the environment (Slater and Bull 1982). In the mixed culture, only *Pseudomonas* strains were able to grow on styrene as the sole source of carbon. The importance of this genus as agents for modifying hydrocarbons to useful products and their role in the biodegradation of several substrates such as natural and synthetic aromatic compounds is well known (Haas 1983).

In chemostat culture the specific activity was increased significantly from 49 $mg \cdot g^{-1} \cdot h^{-1}$ at $D = 0.05\ h^{-1}$ to 293 $mg \cdot g^{-1} \cdot h^{-1}$ at $D = 0.20\ h^{-1}$, which is very high for degradation of such xenobiotic molecules. Furthermore, the capacity of this population to grow on mineral medium without any requirement of added agents (co-metabolites, yeast extract or vitamins) facilitate an eventual industrial application. Due to its high activity 500 $g \cdot m^{-3} \cdot h^{-1}$, this process could be used for decontamination of styrene waste gas.

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